Specific profiles of ion channels and ionotropic receptors define adipose- and bone marrow derived stromal cells

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Specific profiles of ion channels and ionotropic receptors define adipose- and bone marrow derived stromal cells

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A B S T R A C T

Adherent, fibroblastic cells from different tissues are thought to contain subsets of tissue-specific stem/progenitor cells (often called mesenchymal stem cells). These cells display similar cell surface characteristics based on their fibroblastic nature, but also exhibit differences in molecular phenotype, growth rate, and their ability to differentiate into various cell phenotypes. The mechanisms underlying these differences remain poorly understood. We analyzed Ca2+ signals and membrane properties in rat adipose-derived stromal cells (ADSCs) and bone marrow stromal cells (BMSCs) in basal conditions, and then following a switch into medium that contains factors known to modify their character. Modified ADSCs (mADSCs) expressed L-type Ca2+ channels whereas both L- and P/Q-type channels were operational in mBMSCs. Both mADSCs and mBMSCs possessed functional endoplasmic reticulum Ca2+ stores, expressed ryanojine receptor-1 and -3, and exhibited spontaneous [Ca2+]ioscillations. The mBMSCs expressed P2X7 purinoceptors; the mADSCs expressed both P2X (but not P2X7) and P2Y (but not P2Y1) receptors. Both types of stromal cells exhibited [Ca2+]i responses to vasopressin (AVP) and expressed V1 receptor types. Functional oxytocin (OT) receptors were, in contrast, expressed only in modified ADSCs and BMSCs. AVP and OT-induced [Ca2+]i responses were dose-dependent and were blocked by their respective specific receptor antagonists. Electrophysiological data revealed that passive ion currents dominated the membrane conductance in ADSCs and BMSCs. Medium modification led to a significant shift in the reversal potential of passive currents from —40 to —50 mV in cells in basal to —80 mV in modified cells. Hence membrane conductance was mediated by non-selective channels in cells in basal conditions, whereas in modified medium conditions, it was associated with K+-selective channels. Our results indicate that modification of ADSCs and BMSCs by alteration in medium formulation is associated with significant changes in their Ca2+ signaling and membrane properties. © 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Abbreviations: αβγ-MeATP, αβγ-Methyleneadenosine 5′-triphosphate lithium salt; αCSF, artificial cerebrospinal fluid; ADSC, adipose derived stromal cell; AVP, arginine vasopressin; bADSC/bBMSC, ADSC/BMSC in basal conditions; bFGF, fibroblast growth factor-basic; BM, bone marrow; BMSC, bone marrow stromal cell; BzATP, 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium salt; Cm, membrane capacitance; CPA, cyclopiazonic acid; DAP, diaminopimelidole; GABA, γ-aminobutyric acid; GVA, ε-conotoxgin GVA; IκCa, Ca2+-activated K’ channels; Is, transient outward K’ current; IcaL, delayed rectifier K’ current; IR, membrane resistance; mADSC/mBMSC, medium modified ADSC/BMSC; MVIC, ε-conotoxin MVIC; NMDA, N-Methyl-D-aspartic acid; OT, oxytocin; PPADS, pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetraoxadom salt hydrate; RA, retinoic acid; RU, ratio units; Vrest, resting membrane potential; VGCC, voltage-gated Ca2+ channels.

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1. Introduction

Adherent, fibroblastic cells from different tissues (e.g., from bone marrow, adipose tissue, umbilical cord blood, placenta, Wharton jelly, etc.) are thought to contain subsets of tissue-specific stem/progenitor cells (often called mesenchymal stem cells). These tissue-specific stem/progenitor cells share many biological features. However, they also display differences in molecular phenotype, growth rate, and their ability to differentiate into various phenotypes (Kern et al., 2006; Al-Nbaheen et al., 2013; Choudhery et al., 2013).

Calcium is a ubiquitous intracellular messenger that is a key regulator of the cell cycle, particularly during stem cell proliferation and modification. The Ca$^{2+}$ signaling pathways have been studied in a variety of stem cell types including embryonic (Forostyak et al., 2013; Viero et al., 2014), fetal (Cocks et al., 2013) and adult stromal cells (Resende et al., 2010; Zippel et al., 2012; Kotova et al., 2014; Forostyak et al., 2016).

Bone marrow stromal cells (BMSCs) have been shown to express L-type Ca$^{2+}$ channels (Heubach et al., 2004; Li et al., 2006; Wen et al., 2012), glutamate receptors (Fox et al., 2010) and have been reported to generate spontaneous inositol 1,4,5-triphosphate (InsP$_3$)-dependent Ca$^{2+}$ oscillations (Kawano et al., 2002, 2003). Adipose tissue-derived stem cells (Karnawa et al., 2002, 2003) express tissue-specific Ca$^{2+}$ channels (ADSCs), were found to express adrenoreceptors, InsP$_3$ receptors (InsP$_3$Rs), purinoreceptors and were reported to generate Ca$^{2+}$-induced Ca$^{2+}$ release (Kotova et al., 2014). BMSCs have also been shown to express specific K$^+$ channels including Ca$^{2+}$-activated K$^+$ channels (kCa), delayed rectifier K$^+$ current (I$_{KDR}$), and transient outward K$^+$ current (I$\text{to}$) (Li et al., 2016). In human BMSC in basal conditions (bBMSCs) large conductance Ca$^{2+}$-activated K$^+$ channels have been identified (Heubach et al., 2004). To the best of our knowledge, an in depth analysis of ion channels and receptors in ADSCs and BMSCs that have been harvested under the same environmental conditions has not been performed. In this study, we compared the functional properties of these two types of cells in basal conditions (bADSCs and bBMSCs) and after their modification (mADSCs and mBMSC) induced by switching to a medium containing factors known to alter their characteristics.

2. Experimental procedures

2.1. Animals

All experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) regarding the use of animals in research, and were approved by the Ethics Committee of the Institute of Experimental Medicine, Academy of Sciences of the Czech Republic (ASCR), Prague, Czech Republic. The Sprague–Dawley rats were housed under standard laboratory conditions: a 12:12 h dark:light cycle, at 23 °C, with food and water supplied ad libitum. Bone marrow and adipose tissues used for cell isolation were collected from animals that were adequately anesthetized and subsequently euthanized.

2.2. Isolation of ADSCs

The isolation of stromal cells from adipose tissue was performed according to the protocol described previously (Arboleda et al., 2011). Adipose tissue from the inguinal pads was dissected, mechanically minced and treated with 0.2% (w/v) collagenase type I (Warthington Biochemicals, Lakewood, NJ) for 1 h at 37 °C. The isolated cellular fraction was re-suspended in a proliferation medium, consisting of Dulbecco’s modified Eagle’s medium-DMEM/F12 + Glutamax (Gibco) supplemented with 10% foetal bovine serum and 0.2% antibiotics (primocin), and was then plated into culture flasks. Cells were harvested once they reached 90% confluence and re-plated up to the second passage. Cells from the second passage were used in their basal condition (bADSCs) or after growth in modified medium for further studies.

2.3. Isolation of BMSCs

As described previously (Forostyak et al., 2011), bone marrow (BM) was taken from femurs and tibias of 16-day-old rats. After cutting the epiphysis, BM was washed from the bones using a 2-ml syringe with a 21-gauge needle filled with DMEM containing high glucose, Glutamax 15 µM (Gibco), 10% fetal calf serum and primocin 0.2%. The BM was gently dissociated and then plated into Petri dishes. The medium was changed after 24 h. When cells reached 75–90% confluence, they were detached by trypsin/EDTA treatment and transferred into culture flasks. Cells were used in their basal condition (bBMSCs) or after growth in modified medium for further studies.

2.4. Medium-modified ADSCs and BMSCs

Cultured bBMSCs or hADSCs (passage 2), after reaching 75–90% confluence, were plated at a density of 1 × 10³ cells on glass bottom Petri dishes. After the attachment of the cells, the culture medium was replaced with medium consisting of a Neurobasal medium with B27 supplements containing retinoic acid (RA), 40 ng/ml, fibroblast growth factor-basic (bFGF) and 1% primocin. The cells were exposed to B27 with RA for 72 h, and then the culture media were replaced with Neurobasal medium containing B27 supplements (without RA), 40 ng/ml, bFGF and 1% primocin, and kept in culture up to 1 week. Growth factors were added every second day. The cells were measured between day 3 and day 5. This process was selected based on previous studies that explored the possibility to differentiate ADSCs into neuronal cells. Although changes in gene expression were noted, differentiation into functional neurons was not achieved (Arboleda et al., 2011).

2.5. Measurements of [Ca$^{2+}$]$_i$ using the fast fluorescence photometry system

[Ca$^{2+}$]$_i$, measurements on single cells were performed according to previously reported methods (Dayanithi et al., 1996; Forostyak et al., 2013). The cells were plated on 24 mm glass-bottom dishes (WillCo Dishes BV, Amsterdam, Netherlands) coated with laminin (Sigma-Al drich), were incubated with 2.5 µM Fura-2 AM (Invitrogen, Carlsbad, CA, USA) with 0.02% Pluronic F-127 (Molecular Probes, Eugene, OR, USA) in culture medium at 37 °C and 5% CO$_2$ for 40 min. Loaded cells were then washed and the culture medium replaced with normal Locke’s buffer containing (in mM): NaCl, 140; KCl, 5; MgCl$_2$, 1.2; CaCl$_2$, 2.2; glucose, 10; HEPES-Tris, 10; pH 7.25, osmolality 298–300 mosmol/l$^{-1}$ and kept at 37 °C throughout the time course of the experiment. Fluorescence measurements of [Ca$^{2+}$+], were performed with a fast fluorescence microspectrofluorimeter system based on an inverted microscope (Axiovert, Zeiss-Germany) equipped for epifluorescence (Plan-Neofluar 100×/1.30 oil immersion objective). To achieve fast switching between different excitation wavelengths, a rotating filter wheel was mounted in the excitation light path. The cells were illuminated (200 Hz) alternately at 340 ± 10 and 380 ± 10 nm. In order to minimize the background noise of the Fura-2 signal, successive values were averaged to a final time resolution of 320 ms. The measuring/recording amplifier was synchronized to the filter wheel to measure the fluorescence intensities resulting from different wavelengths. The FFP software controlled the acquisition of the intensity data and provided functions for adjusting the signal values as well as the display and storage of the measured data. A CCD camera was used to visualize the cells. The [Ca$^{2+}$+], measurement values are expressed as the ratio units (RU) between the fluorescence obtained with two excitation wavelengths, 340 nm (A) and 380 nm (B). Fura-2 calibration was performed in these cells in vitro following the procedure described previously (Lambert et al., 1994; Komori et al., 2010; Forostyak et al., 2013), which yielded R$_{min}$ = 0.08, R$_{max}$ = 2.02, β = 1.757. The dissociation constant for Fura-2 at 37 °C was assumed as K$_D$ = 224 nM.
2.6. [Ca$^{2+}$]$_i$ measurements using CCD video-imaging system

[Ca$^{2+}$]$_i$ measurements on several cells were performed using video imaging system with an Axio Observer D1 (Zeiss) inverted microscope equipped with epifluorescence oil immersion objectives (Plan Neofluar 100× 1.30, FLUOR 40×/1.3 oil and FLUOR 20×0.75, Zeiss). The excitation light from a Xenon lamp passed through a Lambda D4 ultra-fast wavelength switching system (Sutter Instruments) with a maximum switching frequency of 500 Hz. The fluorescence intensity was detected by using a cooled CCD camera (AxioCam MRm, Zeiss) and the whole system was controlled by Zeiss ZEN Imaging software (2012-SP2/AxioVision SE64 Rel. 4.8.3). The fluorescence intensity was measured with excitations at 340 and 380 nm, and emission at 510 nm.

2.7. Drugs and solutions

Chemicals were obtained from the following companies: Sigma-Aldrich (St. Louis, MO, USA): cadmium chloride, nickel chloride, nicardipine hydrochloride, ATP, αβ-Methyleneadenosine 5′-triphosphate lithium salt (αβ-MeATP), pyridoxal phosphate-6-azo[benezene-2,4,6-4-disulfonic acid], tetrasodium salt hydrate (PPADS), 2′(3′)-O-[(4-benzozybenzoyl)adenosine 5′-triphosphate triethylammonium salt (BeATP), KN-62, γ-glutamatic acid potassium salt monohydrate, N-Methyl-t-aspartic acid (NMMA), α,γ-diaminobutyric acid (GABA), adenosine, oxytocin acetate salt hydrate (OT), [Arg$^1$]-vassopressin acetate salt (AVP), and [deamino-Pen$^1$, O-Me-Tyr$^2$, Arg$^8$]-Vasopressin; Tocris Bioscience (Bristol, UK): MRS2179; NF279; Alomone Labs Ltd. (Jerusalem, Israel): ryanoidine, cyclopiazonic acid (CPA), α- or β-homo MVIC (MVIC), α- or β-homo GVIA (GVIA); Phoenix Pharmaceuticals Inc.: [d(Glu)$^2$]-Tyr[Me]$^2$ (OMT$^2$) vasotocin; [d(Glu)$^2$]-OVT]. Concentrated stock solutions of nicardipine, glutamate, KN-62 and ryanoidine were prepared in DMSO, while the remaining stock solutions of agonists/antagonists were dissolved in dH$_2$O. All concentrated stock solutions were stored at −20 °C. Test solutions were prepared daily using aliquots from frozen stocks to obtain the working concentrations. All buffers and solutions in this study were made explicitly using ion-free dH$_2$O from Merck-Germany.

2.8. Drug application

As described previously (Dayanithi et al., 2006; Viero et al., 2006; Forostyak et al., 2013), the control and test solutions were applied using a temperature controlled multichannel polypropylene capillary perfusion system 3 (Warner Instruments, Inc., USA). The temperature of all solutions was maintained at 37 °C. After each application of the tested drug, the cells were washed with control buffer. This method allowed for fast and reliable exchange of the solution surrounding the selected cell under observation without exposing the neighboring cells.

2.9. Patch-clamp recordings

Cell membrane currents were recorded 3–4 days after the onset of differentiation using the patch-clamp technique in the whole-cell configuration. Recording pipettes with a tip resistance of 8–10 MΩ were made from borosilicate capillaries (0.86 ID, Sutter Instruments Company, Novato, CA, USA) using a P-97 Brown-Flaming micropipette puller (Sutter Instruments, Novato, CA, USA). Recording pipettes were filled with a solution containing (in mM): KCl 130, CaCl$_2$ 0.5, MgCl$_2$ 2, EGTA 5, HEPES 10. The pH was adjusted with KOH to 7.2. To visualize the recorded cells, the intracellular solution contained Alexa-Fluor hydrazide 594 (Molecular Probes, Carlsbad, CA, USA). The labeled cells were used for further post-recording immunocytochemical identification. All recordings were recorded in artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 122, KCl 1.5, MgCl$_2$ 1.3, Na$$_2$$HP0_4$$^2$ 1.25, NaHCO$_3$ 28, d-glucose 10, osmolarity 300 ± 2 mosmol/l$^{-1}$. The solution was continuously gassed with a mixture of 95% O$_2$ and 5% CO$_2$ to maintain a final pH of 7.4. All recordings were made on cover slips perfused with aCSF at room temperature. Electrophysiological data were measured with 10 kHz sample frequency using an EPC10 amplifier controlled by PatchMaster software (HEKA Elektronik, Lambrecht/Pfalz, Germany) and were filtered using a Bessel filter. The coverslips with cells were transferred to the recording chamber of an upright Axioskope microscope (Zeiss, Göttingen, Germany) equipped with electronic micromanipulators (Luigs & Neu mann, Ratingen, Germany) and a high-resolution AxioCam HRc digital camera (Zeiss, Göttingen). The resting membrane potential ($V_{m}$) was measured by switching the EPC-10 amplifier to the current-clamp mode.

The membrane resistance (IR) was calculated from the current elicited by a 10 mV test pulse depolarizing the cell membrane from the holding potential of −70 mV to −60 mV for 50 ms, 40 ms after the onset of the depolarizing pulse. Membrane capacitance ($C_m$) was determined automatically from the Lock-in protocol by PatchMaster. Current patterns were obtained by 50 ms hyper- and depolarizing the cell membrane from a holding potential of −70 mV to values ranging from −160 mV to +40 mV, at 10 mV intervals (Androsova et al., 2006; Nepravská et al., 2007). Electrophysiological data were analyzed using Fitmaster software (HEKA, Lambrecht, Germany). Membrane potentials were corrected for the liquid junction potential using CPCalcCW software (Barry, 1994). After recording, the coverslips were fixed in phosphate buffer (0.2 M PB, pH 7.4) containing 4% paraformaldehyde for 15 min and then transferred to PBS (10 mM, pH 7.2).

2.10. Antibodies and immunocytochemistry

Cells plated onto laminin-coated coverslips were fixed and immunostained according to the protocol described previously (Forostyak et al., 2013). The primary and secondary antibodies used in the study are listed in Table 1. For each experiment a negative control was performed: following the same protocol, cells were blocked with normal goat serum and incubated only with secondary antibodies. To visualize the cell nuclei, following immunostaining the coverslips were incubated with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 5 min at RT (24 °C), mounted using Aqua Poly/Mount and examined using a ZEISS LSM 510 DUO confocal microscope.

2.11. Data analysis and statistical methods

Origin 8.5.1 was employed for plotting and statistical procedures. The results are expressed as mean ± SEM. The sample size (n) given is the number of cells tested according to the same protocol (control, test drug, recovery) for each group. The figures (traces) show on-line single cell measurements of the [Ca$^{2+}$]$_i$ levels before and after the application of test substances, while bar diagrams and numerical data are corrected for the liquid junction potential using JPCALCW software (HEKA Elektronik, Lambrecht/Pfalz, Germany) and were filtered with a Bessel filter.

3. Results

3.1. [Ca$^{2+}$]$_i$ dynamics

The functional properties were studied using a minimum of 4 and a maximum of 10 independent cell culture preparations for both ADSCs and BMSCs. The resting level of [Ca$^{2+}$]$_i$ in bADSCs was 303 ± 8 nM, maximum of 10 independent cell culture preparations for both ADSCs and BMSCs. The resting level of [Ca$^{2+}$]$_i$ in bADSCs was 303 ± 8 nM, p = 0.012 and became similar to bADSCs. In contrast, the resting [Ca$^{2+}$]$_i$ level in aBMSCs was significantly (p = 0.012) higher (332 ± 4 nM, n = 26) compared with bADSCs, although after medium modification, it decreased to 300 ± 8 nM, n = 49, p = 0.012 and became similar to bADSCs.

In bADSCs and bBMSCs, changes in [Ca$^{2+}$]$_i$ were monitored in response to a high K$^+$ concentration (50 mM), glutamate (100 μM), ATP...
(100 μM), cyclopiazonic acid (CPA, 10 μM), oxytocin (OT, 100 nM and 1000 nM) and vasopressin (AVP, 100 nM). Both bADSCs and bBMSCs were sensitive to ATP and vasopressin, but not to the inhibitory neurotransmitters (glutamate and GABA), oxytocin or to depolarization by K+ (Table 1). bBMSCs, but not bADSCs, were sensitive to 10 μM CPA, sarcoplasmic reticulum Ca2+ -ATPase pump inhibitor. A representative trace, showing a typical [Ca2+]i response to various agonists in bBMSCs is shown in Fig. 1.

3.2. Glutamate receptors

Both bADSCs and bBMSCs did not respond to 100 μM glutamate, suggesting the absence of functional glutamate receptors (Fig. 1). Likewise the application of glutamate in mBMSCs had no effect, only one cell out of 11 showed a weak response to 100 μM glutamate. Only in mADSCs (4 out of 15 cells, 27%) glutamate at 100 mM concentration evoked a rapid [Ca2+]i increase with a mean amplitude of 1.02 ± 0.22 RU. Application of glutamate in mBMSCs had no effect, only one cell out of 15 showed a weak response to 100 mM glutamate. Application of glutamate in bBMSCs and bADSCs was not effective, only one cell out of 14 (7%) showed a weak response.

3.3. Voltage-gated Ca2+ channels

An influx of Ca2+ through voltage-gated Ca2+ channels (VGCC) is typical for excitable cells. bBMSCs and bBMSCs did not respond to depolarization by 50 mM K+, suggesting the absence of functional VGCC in basal conditions (Table 1, Fig. 1), while after medium modification a subpopulation [46% of mADSCs (18 out of 39 cells) and 42% of mBMSCs (17 out of 41)] responded to the application of 50 mM K+ by a rise in [Ca2+]i (Table 1). The mean amplitude of this [Ca2+]i increase was 1.13 ± 0.17, n = 18 in mADSCs and 0.5 ± 0.12, n = 17 in mBMSCs. Pre-incubation with Cd2+ (100 μM), a non-specific blocker of high-voltage activated Ca2+ channels, together with Ni2+ (50 μM), a blocker of low-voltage activated Ca2+ channels, for 5 min completely blocked [Ca2+]i responses in 4 out of 5 tested mADSCs, while in the remaining cell, the K+-induced [Ca2+]i increase was inhibited by 34% (Fig. 2C, D). Similarly, pre-incubation of mBMSCs with nicardipine effectively blocked [Ca2+]i responses in all 4 tested cells, suggesting the role for L-type Ca2+ channels (Fig. 2C, E). A specific P/Q-type blocker, ω-conotoxin MVIIC applied at 300 nM significantly inhibited (98% ± 0.3%) the spontaneous Ca2+ oscillations (Fig. 3I). The mean amplitude of the spontaneous Ca2+ oscillations by 53 ± 12%, p = 0.001, n = 6. Immunocytochemical staining revealed positive staining for L-(Fig. 2H, J, L) and P/Q-types (Fig. 2I, K, L), but not N-type VGCC (Fig. 2L) both in mADSCs and mBMSCs.

3.4. Ca2+ release from intracellular stores and spontaneous [Ca2+]i oscillations

To check the functional role of intracellular Ca2+ stores, we used a reversible inhibitor of sarco-endoplasmic reticulum Ca2+-ATPase pump, CPA, at 10 μM. None of the 14 tested bADSCs responded to CPA, while after medium modification all 4 mADSCs tested responded to CPA by a rise of [Ca2+]i, with the mean amplitude of 0.62 ± 0.08 (Table 1). In contrast, both bBMSCs and mBMSCs were sensitive to CPA: 80% (16 out of 20) of bBMSCs and 92% (11 out of 12) of mBMSCs generated [Ca2+]i increase (Table 1). The mean amplitude of [Ca2+]i increase was 0.36 ± 0.05 (n = 16) in bBMSCs and 0.41 ± 0.04 (n = 11) in mBMSCs, respectively. Application of 2 μM ryanodine (which at this concentration activates RyRs) (Lanner et al., 2010) caused a [Ca2+]i rise in 63% of the mADSCs (5 out of 8), with a mean amplitude of 1.39 ± 0.3. Immunostaining against ryanodine receptor subtype 1 showed that bADSCs expressed all three types (RY1, RY2 and RY3) of receptors, while bBMSCs expressed mostly RY1 and RY3 with only a few cells positive for RY2 (Fig. 3A). The expression of RY1 (Fig. 3A, B, E) and RY3 (Fig. 3A, D, G) remained unchanged or even increased in mADSCs and mBMSCs, while the expression of RY2 in mADSCs decreased and was undetectable in mBMSCs (Fig. 3A, C, F).

Both mADSCs and mBMSCs exhibited spontaneous [Ca2+]i oscillations, although only 12% of the bADSCs and none of the bBMSCs showed spontaneous [Ca2+]i oscillations. A subpopulation of mADSCs (11 out of 83 cells; 13%) exhibited [Ca2+]i oscillations (Fig. 3H); these were maintained after the removal of extracellular Ca2+. The mean amplitude of the spontaneous [Ca2+]i transients in mADSCs was 0.97 ± 0.15, the mean duration was 85.5 ± 19 s, and they appeared with a mean frequency of 4.76 mHz. About 29% of mBMSCs (14 out of 49) exhibited irregular oscillations (Fig. 3I). The mean amplitude of the spontaneous [Ca2+]i transients in mBMSCs was 0.6 ± 0.14, the mean duration was 59.7 ± 9 s, and they appeared at a mean frequency of 6.94 mHz. In contrast to mADSCs, these oscillations were significantly inhibited by the application of non-specific VGCC blockers, 100 μM Cd2+ and 50 μM Ni2+ which decreased the mean amplitude of oscillations by 53 ± 12%, p = 0.001, n = 6.

3.5. Purinergic receptors

The majority of cells in basal conditions [90% (n = 22) of bADSCs and 62% (n = 26) of bBMSCs], exhibited [Ca2+]i transients in response to the purinergic receptor agonist, 100 μM ATP (Table 1). The mean amplitude of [Ca2+]i increase in response to 100 μM ATP in bADSCs was 2.1 ± 0.16, n = 20 and in bBMSCs was 0.83 ± 0.16, n = 16. The increase in [Ca2+]i in all bBMSCs tested was significantly inhibited (98% ± 0.3%) by a non-selective P2 receptor antagonist, 10 μM PPADS (p = 0.0004, n = 5; Fig. 4A). On the contrary, in the majority of bADSCs (88%), PPADS had no effect (Fig. 4A), and only in 12% of cells tested, the ATP-

Table 1

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<th>ADSCs</th>
<th>BMSCs</th>
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<tr>
<td></td>
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<td>100%</td>
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induced $[\text{Ca}^{2+}]_i$ rise was inhibited by 47% ± 4%. Other P2X antagonists KN-62 and NF279 had no effect in both bADSCs and bBMSCs.

Application of 100 μM ATP in mADSCs induced a rapid increase in $[\text{Ca}^{2+}]_i$ with a mean amplitude of 1.55 ± 0.09 in all 43 cells tested (Fig. 4B). BzATP at a 20 μM concentration (Fig. 4B) appeared to be a less potent agonist than ATP, inducing a significantly smaller increase in $[\text{Ca}^{2+}]_i$ of 0.92 ± 0.12 in 88% of cells tested (14 out of 16; p = 0.001). In 24% (4 out of 17) of the cells, the least potent agonist, αβ3-meATP (100 μM), caused a significantly weaker $[\text{Ca}^{2+}]_i$ response compared to ATP with a mean amplitude of 0.7 ± 0.04 (p = 0.01; Fig. 4B). The application of adenosine (100 μM) had no effect. The application of 100 μM ATP in the absence of extracellular $\text{Ca}^{2+}$ caused the most potent activation of purinergic receptors, significantly higher compared to BzATP (p = 0.001) and αβ3-meATP (p = 0.01), but not to ATP (p = 0.4). ATP at low $\text{Ca}^{2+}$ induced a $[\text{Ca}^{2+}]_i$ increase of 1.87 ± 0.49 in all 4 tested cells. Increases in $[\text{Ca}^{2+}]_i$, induced by αβ3-meATP were significantly (by 98 ± 0.4%) inhibited by pre-incubation with PPADS in all 4 cells tested (Fig. 4C). Neither P2X7-selective antagonist NF279 (1 μM) nor P2X7-selective antagonist KN-62 (1 μM) affected BzATP-induced $[\text{Ca}^{2+}]_i$ increase (p = 0.7, n = 6). Another antagonist, MRS2179, selective for P2Y1 receptors also did not affect the ATP-induced $\text{Ca}^{2+}$ responses (p = 0.28, n = 4).

The number of mBMSCs responsive to ATP increased (86%) when compared with bBMSCs (62%). mBMSCs were also sensitive to other agonists of purinergic receptors. Unlike mADSCs, where ATP was the most potent activator of purinoceptors, in mBMSCs the highest $[\text{Ca}^{2+}]_i$
responses were induced by BzATP. The amplitude of 20 μM BzATP-induced [Ca^{2+}] increase was 1.69 ± 0.14, n = 15, significantly higher compared to 100 μM ATP (0.94 ± 0.08, n = 25, p = 0.00005) and to 100 μM αβ3-meATP (0.64 ± 0.16, n = 6, p = 0.0003; Fig. 4B). Application of PPADS, a non-selective antagonist of P2X and P2Y2 receptors, had no effect (p = 0.94, n = 7). KN-62 (1 μM), a selective P2X receptor antagonist, completely blocked BzATP-induced [Ca^{2+}] increase in all cells tested (p = 0.01, n = 4) (Fig. 4D). Expression of P2X-R was also confirmed by immunocytochemical staining (Fig. 4E).

3.6. Vasopressin and oxytocin receptors

Application of 100 nM the neuropeptide vasopressin (AVP) increased [Ca^{2+}] in 75% of tested ADSCs (n = 12) and all BMSCs (n = 19) with a mean amplitude of 1.97 ± 0.3 and 1.36 ± 0.1, respectively (Table 1). In order to test the specificity of AVP action, we used a selective AVP-V1 receptor antagonist, [deamino-Pen1, O-Me-Tyr2, Arg8]-vasopressin at 1 μM. Pre-incubation of cells with AVP-V1 antagonist completely blocked the AVP (100 nM)-induced [Ca^{2+}] response in all ADSCs and in 44% of BMSCs. In the remaining 56% of BMSCs, it suppressed the AVP (100 nM)-induced [Ca^{2+}] response by 40 ± 4.8%, n = 4 (p = 0.036; Fig. 5A). On the other hand, neither ADSCs nor BMSCs responded to another neuropeptide, oxytocin (OT), applied at concentrations of 100 nM or 1000 nM.

Unlike cells in basal conditions, which were sensitive to AVP but not to OT, mADSCs and mBMSCs were sensitive to both OT and AVP. OT at 1000 nM induced an increase in [Ca^{2+}] in 87% of the mADSCs and 73% of mBMSCs tested (Table 1). This response did not desensitize during several sequential applications of OT (Fig. 5D, F) and was concentration-dependent (Fig. 5B). The mean amplitude of the [Ca^{2+}] response to various OT concentrations in mADSCs ranged, respectively, from 0.23 ± 0.03, n = 4 for 10 nM, 0.95 ± 0.3, n = 4 for 500 nM and 1.22 ± 0.19, n = 13, for 1000 nM (Fig. 5B). The amplitudes of the [Ca^{2+}] increase in mBMSCs were: 10 nM OT elicited a [Ca^{2+}] rise of 0.32 ± 0.07, n = 9; 100 nM OT, 0.18 ± 0.04, n = 4; 500 nM OT, 0.59 ± 0.14, n = 6; and 1000 nM OT, 1.02 ± 0.18, n = 8 (Fig. 5B). Incubation with 1 μM dBcAMP, an OT receptor antagonist, completely blocked the OT-induced [Ca^{2+}] rise in both mADSCs (n = 4, Fig. 5H) and mBMSCs (n = 4, Fig. 5I). Immunocytochemical staining with an oxytocin antibody confirmed the presence of OT in the cultured mADSCs (Fig. 5L) and mBMSCs (Fig. 5N).

Application of AVP evoked reproducible (Fig. 5E, G) and dose-dependent (Fig. 4C) increases in [Ca^{2+}] in 94% of the mADSCs and 73% of mBMSCs tested (Table 1). The mean amplitude of [Ca^{2+}] increase in mADSCs was 2.31 ± 0.23, n = 5 at 10 nM AVP; 2.17 ± 0.18, n = 17 at 100 nM AVP; 1.26 ± 0.31, n = 4 at 500 nM AVP and 1.16 ± 0.25, n = 6 for 1000 nM AVP (Fig. 5C). In mBMSCs the amplitude of [Ca^{2+}] increases was 1.03 ± 0.14, n = 7 in response to 100 nM AVP; 0.91 ± 0.17, n = 6 in response to 500 nM AVP and 0.63 ± 0.07, n = 7 in response to 1000 nM AVP (Fig. 5C). Unlike OT, which in high concentrations caused a higher [Ca^{2+}] increases, AVP when applied at a high dose inhibited AVP receptors. Application of V1 selective antagonist [deamino-Pen1, O-Me-Tyr2, Arg8]-vasopressin completely blocked AVP-induced [Ca^{2+}] increases in all 9 mADSCs tested (Fig. 5I) and significantly inhibited by 95 ± 0.7% in all 5 tested mBMSCs (p = 0.0005, Fig. 5K), confirming that functional AVP-V1 receptors are present in all ADSCs and BMSCs. Immunocytochemical staining with a vasopressin antibody revealed the presence of AVP both in mADSCs (Fig. 5M) and mBMSCs (Fig. 5O).

3.7. Membrane properties of ADSCs and BMSCs

The passive membrane properties of ADSCs and BMSCs are listed in Table 2. Most notable, mADSCs and mBMSCs exhibited a significant hyperpolarizing shift of resting membrane potential. In the voltage-clamp settings bADSCs and bBMSCs displayed, in response to de- and hyperpolarizing test pulses, symmetrical currents that decayed during the voltage steps. These currents had a linear current–voltage relationship with E_{rev} at −33.2 ± 4.9 mV (n = 26) for bADSCs and −57.1 ± 8.7 mV (n = 23) for bBMSCs (Fig. 6A, B). An increase in extracellular K+ from 3 mM to 30 mM caused a positive shift in reversal potential in bADSCs by 14 ± 6.7 mV (n = 6) and in bBMSCs by 28 ± 3.1, mV (n = 7). In medium modified cells, voltage pulses similarly evoked symmetric currents, which however were mainly time-independent. The voltage–current relation for these currents was linear with reversal potential at −71.8 ± 1.4 mV (n = 30) for p ADSCs and −71.5 ± 1.5 mV (n = 22) for mBMSCs (Fig. 6C, D). Both, bADSCs and bBMSCs expressed vimentin, the marker of progenitor cells (Fig. 6E).

4. Discussion

In this study, we investigated Ca^{2+} signaling and electrophysiological properties of rat stromal cells obtained from adipose tissue and bone marrow. We analyzed the changes of functional properties under the same environmental conditions upon medium modification that were previously reported to elicit changes in ADSCs (Willingham and Pasant, 1975). We demonstrated that after medium modification, both ADSCs and BMSCs undergo a significant change in their membrane properties and in the expression of channels and receptors associated with generation of Ca^{2+} signals (Fig. 7). The resting [Ca^{2+}], level in mBMSCs significantly decreased after pre-differentiation and became almost the same as in ADSCs. The resting [Ca^{2+}], level in ADSCs remained unchanged after medium modification.

4.1. Voltage-gated Ca^{2+} channels

VGCCs were found in several preparations of stem cells. High voltage activated L-type Ca^{2+} channels were shown to enhance proliferation and osteogenic differentiation in rat BMSCs (Wen et al., 2012). Small dihydropyridine-sensitive currents were recorded in minor subpopulations of undifferentiated BMSCs from humans and rats (Kawano et al., 2002; Heubach et al., 2004; Li et al., 2005, 2006). We were not able to detect any VGCCs-mediated Ca^{2+} entry in BMSCs in basal conditions. This could be explained by differences in the techniques and culture conditions. However, after medium modification, almost half of the cells (42%) were sensitive to depolarization with 50 mM K+. In our experiments, nicardipine completely blocked high K+-induced [Ca^{2+}], responses confirming the functional expression of L-type Ca^{2+} channels both in mADSCs and mBMSCs (Fig. 2). In mBMSCs, α-conotoxin MVIIC, a selective P/Q-type Ca^{2+} channel blocker, significantly inhibited K+-induced [Ca^{2+}], increase, suggesting the additional activation of P/Q-type channels. To our knowledge, this is the first report showing the functional P/Q-type Ca^{2+} channels in rats BMSCs. We were not able to detect any N-type Ca^{2+} channels in BMSCs, either functionally or at the protein expression level. In contrast, several studies demonstrated the (mRNA and protein) expression of CACNA 1 C (L-type) and CACNA 1 G (T-type) in ADSCs in basal and medium-modified conditions (Safford et al., 2004; Bai et al., 2007; Jang et al., 2010). Immunocytochemically, the presence of both the CACNA 1 C subunit of L-type Ca^{2+} channels and the CACNA 1 A subunit of P/Q-type Ca^{2+} channels was detected in mADSCs and mBMSCs.

In summary, depolarization of bBMSCs and bADSCs did not produce [Ca^{2+}], transients, likely indicating the absence of functional VGCCs. Conversely, almost half of the population of mBMSCs (42%) and mADSCs (46%) generated a [Ca^{2+}], increase in response to depolarization. mBMSCs and mADSCs expressed functional L-type Ca^{2+} channels and a small population of mBMSCs (but not mBMSCs) expressed P/Q-type Ca^{2+} channels as well.

4.2. Spontaneous oscillations and intracellular Ca^{2+} stores

Spontaneous [Ca^{2+}], oscillations contribute to many cellular processes, such as secretion, fertilization, etc. (Berridge et al., 2000; Ye, 2010).
In our study, similarly to that of Ichikawa et al. (Ichikawa and Gemba, 2009), we did not observe spontaneous oscillations in BMSCs in basal conditions, although 29% of mBMSCs exhibited spontaneous $[\text{Ca}^{2+}]_{\text{i}}$ activity. Exposure to non-selective VGCC antagonists, Cd$^{2+}$ and Ni$^{2+}$, significantly inhibited the amplitude of spontaneous $[\text{Ca}^{2+}]_{\text{i}}$ increases, but did not block them completely, suggesting the role for intracellular activity.

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"-" - negative immunostaining
"+" - faint positive immunostaining / few cells express the marker
"++" - positive immunostaining / majority cells express the marker
Ca\textsuperscript{2+} stores and VGCC; these findings are in accordance with previous reports (Kawano et al., 2002, 2003). Spontaneous [Ca\textsuperscript{2+}]i oscillations were also observed in a subpopulation of human ADSCs; although the numbers of responding cells differed remarkably from 2 to 5\% (Kotova et al., 2014) to 70\% (Sauer et al., 2011). In our case, in rat ADSCs spontaneous [Ca\textsuperscript{2+}]i dynamics were observed only in a small subpopulation (12\% of bADSCs and 13\% of mADSCs). These spontaneous [Ca\textsuperscript{2+}]i oscillations were not inhibited after removal of extracellular Ca\textsuperscript{2+}, suggesting that intracellular [Ca\textsuperscript{2+}]i stores underlie these oscillations. In addition, mADSCs were sensitive to the application of 2 \(\mu\text{M}\) ryanodine and 10 \(\mu\text{M}\) CPA, suggesting that mADSCs possess functional ER Ca\textsuperscript{2+} stores and ryanodine receptors.

4.3. Purinergic receptors

Purinergic signaling plays an important role during stem cell development, influencing proliferation and determining cell fate, although the effects of agonists depend on the receptor subtype (Forostyak et al., 2013). For example ATP, acting through P2X receptors, induces the proliferation of human hematopoietic stem cells (Glaser et al., 2012). BMSCs at early passages (P0–P5) spontaneously release ATP and inhibit cell proliferation. Inhibition of P2Y1 receptors led to increased proliferation (Coppi et al., 2007); the same P2Y1 receptors in human BMSCs also contributed to InsP3-induced spontaneous [Ca\textsuperscript{2+}]i oscillations (Kawano et al., 2006). Here we also investigated the
Fig. 5. Oxytocin and vasopressin responses in ADSCs and BMSCs in basal and medium modified conditions. A. Bar diagram showing the percentage of the mean peak amplitude of [Ca^{2+}] increase before (AVP control) and after incubation with the 1 μM AVP-V1R antagonist, (d(CH2)5, Tyr(Me)2, Arg8)-vasopressin in bBMSCs and bADSCs (*p = 0.036, n = 4). Control AVP-induced [Ca^{2+}] increase was taken as 100%. B. Dose-dependent [Ca^{2+}] increase in response to application of increasing concentrations OT in mADSCs (blue curve) and BMSCs (green curve). Similarly, C. Dose-dependent [Ca^{2+}] increase in response to the application of increasing concentrations of AVP in mADSCs (blue curve) and BMSCs (green curve). The significance between various agonists among mADSCs and BMSCs is shown by asterisks in blue and green, respectively. Data are mean ± S.E.M. *p<0.05; **p<0.01; ***p<0.001. D–G. Representative traces of an individual mADSCs (D, E) or mBMSCs (F, G) subjected to successive 10 s applications 100 nM OT (D, F) or 100 nM AVP (E, G). H, J. Bar diagrams representing the peak amplitude [Ca^{2+}] (control percentage) response induced by 100 nM OT and a significant inhibition by the presence of OT-antagonist, 1 μM dOVT in mADSCs (H) and BMSCs (J). K. Bar diagrams representing the peak amplitude [Ca^{2+}] (control percentage) response induced by 100 nM AVP and a significant inhibition by the presence of 1 μM AVP-V1R antagonist, in mADSCs (K) and BMSCs (K). L–O. Immunohistochemical detection of OT and AVP in mADSCs and BMSCs. Confocal images showing mADSCs stained for OT (L) and nuclear DAPI staining. Similarly, confocal images showing mBMSCs stained for OT (N) and nuclear DAPI staining. Scale bars = 20 μm.
mBMSCs express P2 receptors, the BMSCs expressed only P2X7 receptors, but not in mADSCs, was also confirmed by immunocytochemical studies (Fig. 4). We conclude that although both mADSCs and mBMSCs express P2 receptors, the BMSCs expressed only P2X7 receptors, ADSCs expressed both P2X (but not P2X7) and P2Y (but not P2Y1) receptors.

4.4. Oxytocin and vasopressin

OT and AVP control a wide range of functions in the central as well as in peripheral nervous systems (Dayanithi et al., 2000, 2008, 2012; Hussy et al., 2001; Ueta et al., 2008; Suzuki et al., 2009; Fujihara et al., 2009; Todoroki et al., 2010; Viero et al., 2010; Moriya et al., 2012, 2015). The primary cultures of neonatal rat cardiomyocytes express OT receptors (Florian et al., 2010). Recently OT has become a subject of increased attention due to its protective and metabolic effects on rats (male Sprague Dawley) BMSCs. Stimulated by pretreatment with OT, BMSCs have been shown to reduce apoptosis, and increase cellular proliferation of ion channel types expressed in tissues of stem/progenitor cells at various differentiated stages require further investigation.

5. Conclusions

Together, as briefly summarized in Fig. 7, our results suggest that the Ca2+ signaling, ion channel and ionotropic receptor expression profile of adult tissue–specific stem/progenitor cells depends not only on the culture conditions but also on the source from which the cells were.
isolated. The fate and functional properties of differentiated cells are driven by intrinsic mechanisms, which are independent of the culture protocol. Identifying their \( \text{Ca}^{2+} \) signaling, ion channel and ionotropic receptor expression profile as a function of external changes in their environment is essential to better utilize these cells in tissue engineering and regenerative medicine.
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Authors’ contributions

O.F. contributed to prepare experimental design, performed Ca^{2+} measurements, helped with data and statistical analysis, immunocytochemistry, confocal imaging, and contributed in manuscript writing.

O.B. performed patch-clamp experiments, helped with electrophysiological data analysis and related statistics, and contributed in manuscript writing on electrophysiological data.

M.A. supervised patch-clamp experiments, helped with electrophysiological data analysis and related statistics, and contributed in manuscript writing on electrophysiological data.

S.F. helped with preparing cell culture and confocal imaging experiments and imaging analysis.

E.S. participated in experimental design, organized logistics, and participated in manuscript writing.

A.V. provided experimental design, helped with electrophysiological data analysis and related statistics, and interpreting the data, and contributed in manuscript writing and editing.

G.D. provided concept and experimental design, project management, supervised the experiments, checked the data and statistical analysis and interpretation, and wrote the manuscript.

Disclosure of potential conflicts of interest

The authors declare that they have no conflicts of interest.

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