CALCIUM-SENSING RECEPTOR ACTIVATION ENHANCES EXPRESSION OF LIPOLYSIS ACTIVATOR GENES THROUGH CALCIUM-DEPENDENT ATP SECRETION IN WHITE ADIPOCYTES

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Abstract. Using the methods of fluorescence microscopy, Tirf microscopy, inhibitory analysis, immunocytochemistry and PCR, it has been shown that in response to an increase in $[Ca^{2+}]o$ or a CaSR agonist – protamine in the minor population of white adipocytes, Ca^{2+} signals are rapidly generated – a short-term Ca^{2+} increase and Ca^{2+}-oscillations, while in most cells the generation of Ca^{2+} responses occurs after a lag period of varying duration. White adipocyte signals for CaSR activation were completely suppressed in the presence of the selective CaSR antagonist, NPS2143, in both cell populations. When CaSR is activated, a calcium-dependent process of secretion of ATP-containing vesicles occurs, which was also suppressed by NPS2143 and a calcium-dependent secretion blocker tetanus toxin. After a 24-hour exposure to the CaSR activator protamine on white adipocytes, an increase in the level of expression of genes – Lipe, Atgl, Sirt1 and Sirt3, encoding hormone-sensitive lipase, triglyceride lipase, sirtuins 1 and 3, respectively. At the same time, an increase in the expression of these genes was not observed with the selective CaSR antagonist, NPS2143. Thus, one of the new mechanisms of activation of genes regulating white adipose tissue lipolysis can be assumed through an increase in $[Ca^{2+}]i$ in the minor population of CaSR-expressing adipocytes, followed by calcium-dependent ATP secretion and paracrine activation of the entire cell network, which will help play an important role in the regulation of the balance of lipogenesis/lipolysis processes.

Keywords: white adipocytes, calcium-sensing receptor, calcium, lipogenesis, lipolysis, intracellular signaling, secretion, ATP.

List of Abbreviations

CaSR – calcium-sensing receptor
PLC – phospholipase C
IP_3 – inositol 3 phosphate
PLA2 – phospholipase A2
MAPK – mitogen-activated protein kinase
ERK 1/2 – extracellular signal-regulated kinase
JNK – c-Jun N-terminal kinase
D2T – 2 type diabetes

Introduction

Calcium-sensing receptor (CaSR) is a plasma membrane protein consisting of 1078 amino acids and belongs to the superfamily of G-proteins coupled receptors (Theman & Collins, 2009). CaSR is widely expressed in the body and is found in the tissues of the kidneys, bones, intestines, mammary gland, smooth muscle cells of the aorta, placenta, and adipose tissue (Mattar *et al.*, 2020) CaSR activation occurs not only by an increase in the concentration of Ca^{2+} in the extracellular environment ($[Ca^{2+}]o$), but also by the action of Mg^{2+}, polyamines, with a change in the strength of ionic voltage (NaCl concentration), pH and concentration of L-amino acids (Mirnaya *et al.*, 2010).

CaSR plays an important role in maintaining calcium concentration through 2 mechanisms – direct, by regulating the excretion of calcium and water from the body, and indirect, by altering the secretion of paraterioid hormone (Alpern *et al.*, 2008). Moreover, CaSR is capable of both activating and inhibiting both of these mechanisms. It has been established that it is the activation of CaSR, and not the level of parathyroid hormone, that is necessary to maintain the normal level of Ca^{2+} ions in the blood serum (Kantham *et al.*, 2009). In addition to maintaining calcium homeostasis in the body, CaSR regulates a number of cellular functions – proliferation, membrane tension, and apopto-

sis (Tfelt-Hansen & Brown, 2005) It is known that activation of the CaSR gene can have both pro and antiproliferative effects. Moreover, there is compelling evidence that a high level of CaSR expression contributes to the resistance of malignant tumors to chemotherapy and radiation therapy (Chattopadhyay, 2006). At the level of intracellular signaling, with an increase in $[Ca^{2+}]o$ or the action of calcium mimetics on CaSR, PLC is activated through the Gqα- or G11α-subunits of heterotrimeric G-proteins and the production of secondary messengers – IP3 and diacylglycerol (Kifor *et al.*, 1997). In addition to PLC, activation of CaSR can stimulate PLA2 and lead to the formation of arachidonic acid (Handlogten *et al.*, 2001; Kifor *et al.*, 2001). In parallel, CaSR can lead to the activation of phosphatidylinositol 4-kinase (Huang, 2002), which is a key enzyme in the reduction of phosphatidylinositol-4,5-bisphosphate and a regulator of apoptosis and the activity of a number of ion channels (Hofer & Brown, 2003). In a number of cells, CaSR activation is also associated with Giα-subunits, inhibition of adenylate cyclase, and a decrease in the level of intracellular cAMP (de Jesus Ferreira *et al.*, 1998). It is known that CaSRs affect the proliferation of a number of cells by regulating the activity of the MAPK signaling pathway and its key enzymes, ERK 1/2 and JNK (Kifor *et al.*, 2001; Arthur *et al.*, 2000). There is evidence from a variety of approaches that a diet high in calcium leads to a decrease in triglyceride accumulation in white adipocytes and to weight loss. In addition, it has been shown that in response to an increase in calcium in the diet, lipolysis increases, weight loss, and a decrease in fat mass (Zemel *et al.*, 2000). High levels of calcium and vitamin D intake during the morning meal leads to activation of fat oxidation and thermogenesis (Ping-Delfos & Soares, 2011). On the other hand, a decrease in calcium intake leads to an increase in body weight and adipose tissue. There is a hypothesis that in response to a decrease in dietary calcium intake, an increase in the level of parathyroid hormone and 1,25-dihydroxyvitamin D (calcitriol) occurs, which leads to an increase in $[Ca^{2+}]i$ and activates the accumulation of triglycerides in adipocytes (Zemel *et al.*, 2000; Zemel, 2001). Treatment of preadipocytes, differentiated adipocytes and explants of human white adipose tissue with allosteric activators of CaSRs enhances the expression of inflammatory cytokines (Cifuentes *et al.*, 2008).

Thus, there is evidence both in support of the negative effect of CaSRs activation in white adipose tissue, and vice versa, indicating the activation of metabolic and signaling pathways aimed at preventing obesity and D2T. Therefore, a detailed study of the mechanisms of action of CaSR through the regulation of Ca^{2+} signaling of adipocytes is an urgent task.

Methods

Isolation of white pre-adipocytes and culturing of adipocytes. Isolation of preadipocytes was carried out in accordance with the method described earlier (Turovsky *et al.*, 2012). All operations took place under sterile conditions on ice. NMRI mice (3–5 weeks old) were anesthetized with CO_2, subjected to cervical dislocation, and disinfected with 70% ethanol prior to dissection. White adipose tissue was taken from the epididymal depot and placed in a Petri dish with cold DMEM (Sigma). Shredded white adipose tissue was transferred into a tube with sterile DMEM medium containing 7 mg of type II collagenase (Sigma) and 4% bovine serum albumin (BSA, FFA). Then the tissue was incubated at 37°C for 18 min in a water bath. To stop the enzyme activity, the tube was cooled on ice for 20 min with occasional shaking. Then the suspension was filtered through a 250 μm filter and centrifuged at 1000g for 10 minutes. The resulting precipitate was resuspended in cooled DMEM medium, filtered through a 50 μm filter, and the cells were precipitated at 1000g for 10 minutes. The pellet was last resuspended in culture medium containing: DMEM, 10% FBS (Gibco), 4 mM L-glutamine, 4 nM insulin, 0.004% gentamicin, and 25 μg/ml sodium ascorbate (Sigma).

The resulting suspension contained preadipocytes, since mature adipocytes contain fat droplets and do not precipitate under these centrifugation conditions. 100 μl of the resulting suspension was applied to a 25 mm diameter

round coverslip (VWR International) in a Petri dish, and then the preparations were placed in a CO_2-incubator for 5 hours at 37°C for cell attachment. After that, 1.5 ml of culture medium was added to Petri dishes. Every 3 days, the culture medium was replaced with a fresh one. For work, we used a culture of adipocytes at the age of 9 days in vitro (9 DIV).

Cell staining. The concentration of calcium ions in the cytoplasm ($[Ca^{2+}]i$) was assessed using a two-wave probe Fura-2 in accordance with the well-known method (Grynkiewicz *et al.*, 1985). For the staining of adipocytes, Fura-2 AM ester was used at a final concentration of 4 μM in Hanks solution, containing (in mM): 156 NaCl, 3 KCl, 1 MgSO4, 1.25 KH2PO4, 2 $CaCl_2$, 10 glucose and 10 HEPES, pH 7.4. 200 μL of a freshly prepared dye solution was added to each coverslip with cell culture and incubated in a thermostat for 40 min at 37°C. After that, the culture was washed with Hanks solution and incubated for 10–15 min to complete the deesterification of the dye.

Fluorescence measurements. The measurement of cytosolic $[Ca^{2+}]$ was performed by fluorescent microscopy using Fura-2AM (Molecular probes, USA), a ratiometric fluorescent calcium indicator. Cells were loaded with the probe dissolved in Hanks balanced salt solution (HBSS), containing 10 mM HEPES, pH 7.4, at a final concentration of 5 μM at 370C for 40 min with subsequent 15 min washout. The coverslip containing the cells loaded with Fura-2 was then mounted in the experimental chamber. During the experiment we used a perfusion system, which enables complete replacement of the cell bathing solution within 30 seconds. We used an Axiovert 200M based imaging system (Carl Zeiss, Germany) equipped with HBO100 mercury lamp, AxioCam HSm CCD camera and MAC5000 high speed excitation filter wheel. Fura-2 fluorescence was excited at two wavelengths using band-pass filters BP 340/30 and BP 387/15; fluorescence was registered in the wavelength range of 465–555 nm. Excitation light intensity was lowered using 25 and 5% neutral density filters in order to prevent phototoxicity. Image frames were acquired at 3 seconds intervals with a Plan Neofluar 10×/0.3 objective. The time lapse image sequences were analyzed using ImageJ 1.44 (NIH Image, Bethesda, MD). Graphs were plotted using OriginPro 8.0 software Microcal Software Inc., Northampton, MA). Statistical analysis was performed using the same software. Results are presented as means ± standard error (SE) or as representative calcium signal of the cells.

Immunocytochemical staining of adipocytes. CaSR in cells was detected using the immunocytochemical staining method. The cells were fixed with 4% paraformaldehyde solution in PBS for 20 min. This was followed by three 5-minute washing of cells with ice-cold PBS. For permeabilization, cells were incubated for 15 min in 0.1% Triton X-100 solution. Blocking of nonspecific binding of antibodies was performed using a 10% solution of donkey serum in PBS. The cells were incubated in blocking solution for 30 min at room temperature. The primary antibodies were loaded for 12 h at 4 °C. Mouse monoclonal antibodies against CaSR (Santa Cruz Biotechnology, USA), dissolved in 1% donkey serum in a ratio of 1: 500, were used as primary antibodies. After incubation with primary antibodies, the cells were washed three times with PBS with an interval of 5 min. Then the cells were loaded with secondary antibodies, which were donkey antibodies to mouse antibodies conjugated with the Alexa Fluor 488 fluorescent dye (Abcam, UK). Incubation with secondary antibodies was performed at room temperature in the dark for 90 min. Secondary antibodies were dissolved in PBS at a ratio of 1:200. Antibody fluorescence was visualized using an inverted laser scanning confocal microscope Leica TCS SP5 (Leica, Germany). An argon laser with a 488 nm band was used to excite fluorescence. Emission was recorded in the range 505–565 nm. Cell nuclei were stained with a Draq5 probe.

Cell staining with quinacrine. For visualization of ATP-containing vesicles, white adipocytes were stained by incubating the cell culture

with 5 μM quinacrine in Hanks medium supplemented with 10 mM HEPES at 37 °C for 15 min. Quinacrine, an acridine derivative, is a weak base that binds ATP with high affinity (Akopova *et al.*, 2012; Gaidin *et al.*, 2019). Then, the cell culture was washed 5 times with Hanks medium and used to visualize the vesicles using TIRF microscopy.

TIRF microscopy. TIRF microscopy was used to visualize and study the dynamics of the secretion of ATP-containing vesicles in adipocytes stained with quinacrine. For this, an inverted TIRF microscope (IX71, Olympus, Japan) equipped with an immersion oil objective with a high numerical aperture (60×, 1.65 NA) and a high-sensitivity cooled high-resolution camera (Hamamatsu, Japan) was used. A series of images were acquired and analyzed using the Olympus Cell^ tool software (Olympus). Quinacrine fluorescence was excited with an argon laser at a wavelength of 488 nm, while registration was performed in the 500 – 530 nm range. To assess changes in fluorescence intensity, the region of interest (ROI) was selected, in which fluorescent vesicle containing ATP were noted. The decrease in fluorescence intensity in the area of interest indicated the secretion of the vesicle into the extracellular space. The experiments were carried out at 37 °C.

Isolation of total RNA. Total RNA was isolated from a primary culture of white adipocytes using the Mag Jet RNA reagent kit (Thermo Scientific, USA) according to the manufacturer's instructions. RNA quality was assessed by electrophoresis in 2% agarose gel in TBE-buffer in the presence of ethidium bromide (1 μg/ml). The RNA concentration was measured using a NanoDrop 1000c spectrophotometer (Thermo Scientific, United States).

cDNA was synthesized using the RevertAid H Minus First Strand kit according to the manufacturer's protocol (Thermo Scientific). Single-stranded cDNA preparations were used as templates for real-time PCR analysis.

Real-time polymerase chain reaction (RT-PCR). RT-PCR was carried out in 25 μl of a mixture containing: 5 μl qPCRmix-HS SYBR (Evrogen, Russia), 1 μl (0.2 μM) of each primer, 17 μl water, 1 μl cDNA. Amplification was performed in a 48-well Dtlite5 amplifier (DNA-Technology, Russia). At the beginning, denaturation was performed for 5 min at 95 °C, then 40 amplification cycles: denaturation at 95 °C for 30 s, annealing at a primer-specific temperature (60 °C) for 20 s, and elongation at 72 °C – 30 s. Reporter fluorescence was read at the elongation stage at 62 °C.

RT-PCR data were analyzed using the Dtlite software (DNA-Technology, Russia). Three independent RT-PCR experiments were carried out (three independent cell cultures of white adipocytes). In all experiments, each cDNA sample was amplified in triplicate and the mean values were found.

Primers specific for the studied genes were selected using the FAST PCR 5.4 programs and the NCBI Primer-BLAST system, and synthesized by Evrogen (Russia) (Table 1).

Gene expression was normalized to the control GAPDH gene encoding glyceraldehyde 3-phosphate dehydrogenase. The results were calculated in accordance with the standard method (Livak & Schmittgen, 2001).

Statistical analysis. All presented data were obtained from at least three cell cultures from 2–3 different passages. N – number of the experiments and *n* – number of cells. All values are given as mean ± standard error (SE) or as individual Ca^{2+}-responses. The differences between the columns were estimated with paired t-test. The statistical tests were performed with GraphPad Prism 5 software.

Results

Activation of the calcium-sensing receptor causes the generation of Ca^{2+} oscillations in white adipocytes. We have previously shown that activators of various receptors conjugate to G-proteins cause the generation of various types of Ca^{2+} signals in white adipocytes, activating different pathways for the mobilization of Ca^{2+} ions from the endoplasmic reticulum (Turovsky et al., 2012; Turovsky et al., 2011). In response to an increase in $[Ca^{2+}]o$

Table 1

Genes and primers for them

Gapdh	Forward 5'-tccactcacggcaaattcaac-3' Reverse 5'-cggcatcgaaggtggaagag-3'
Lipe	Forward 5'-gagcactacaaacgcaacgagaca-3' Reverse 5'-aaattcagccccacgcaactct-3'
Sitr1	Forward 5'-ctttcagaaccaccaaagcgga-3' Reverse 5'-acagaaaccccagctccagtca-3'
Sitr3	Forward 5'-acctttgtaacagctacatgcacggt-3' Reverse 5'-ccatcacatcagcccatatgtcttc-3'
Igf2	Forward 5'-cctcctggagacatactgtgccac-3' Reverse 5'-tgtctccaggtgtcatattggaagaa-3'
Gja1	Forward 5'- cttcaatggctgctcctcacca-3' Reverse 5'- gctcgctggcttgcttgttgt-3'
Lipe	Forward 5'-gagcactacaaacgcaacgagaca-3' Reverse 5'-aaattcagccccacgcaactct-3'
Sitr1	Forward 5'-ctttcagaaccaccaaagcgga-3' Reverse 5'-acagaaaccccagctccagtca-3'
Sitr3	Forward 5'-acctttgtaacagctacatgcacggt-3' Reverse 5'-ccatcacatcagcccatatgtcttc-3'
Igf2	Forward 5'-cctcctggagacatactgtgccac-3' Reverse 5'-tgtctccaggtgtcatattggaagaa-3'

up to 3 mM, transient Ca^{2+} signals and Ca^{2+} oscillations (mainly) are generated in cultured white adipocytes (Fig. 1a). In this case, adipocytes in culture are clearly subdivided into 2 populations – in the first population, an increase in $[Ca^{2+}]i$ occurs immediately after presentation of a stimulus in 23±8% of cells (Fig. 1a) of the total number of responders, while in the second population of adipocytes there is a lag-period preceding Ca^{2+} signals, which averages 3±1.5 minutes (Fig. 2b). It is known that an increase in $[Ca^{2+}]o$ has physiological effects through the activation of CaSR in cells of various tissues (Mattar et al., 2020). The addition of 30 μg/ml protamine, an activator of CaSR, also induces the generation of Ca^{2+} signals without a lag phase in one adipocyte population (Fig. 1c), and the same cells responded with Ca^{2+} oscillations to an increase in $[Ca^{2+}]o$ up to 3 mM. In the second population of white adipocytes, only single cells responded to CaSR activation by protamine and an increase in $[Ca^{2+}]o$ even after a long lag period (Fig. 1d). Confirmation of CaSR activation under the action of 3 mM Ca^{2+} ions and protamine is the preliminary incubation of white adipocytes with the selective CaSR antagonist NPS2143 (3 μM, Fig. 1e), when Ca^{2+} signals are absent in all cells.

Based on the data presented in Fig. 1, it can be assumed that white adipocytes are heterogeneous in terms of CaSR expression level. To identify adipocytes expressing CaSR, the experimental approach described for brain neurons was applied (Turovskaya et al., 2020). A cover glass with a cell culture of adipocytes was mounted in the experimental chamber, a grid was applied from below with an indelible marker, placed on a fluorescent microscope, and an area with cells was selected in which Ca^{2+} signals were recorded in response to an increase in $[Ca^{2+}]o$ and protamine (Fig. 2b, c). After the experiment, the cells were fixed and loaded with antibodies against CaSR, the region of interest was found on the marker grid, and an image of the fluorescence of the antibodies was obtained on a confocal microscope (Fig. 2a). The Ca^{2+}

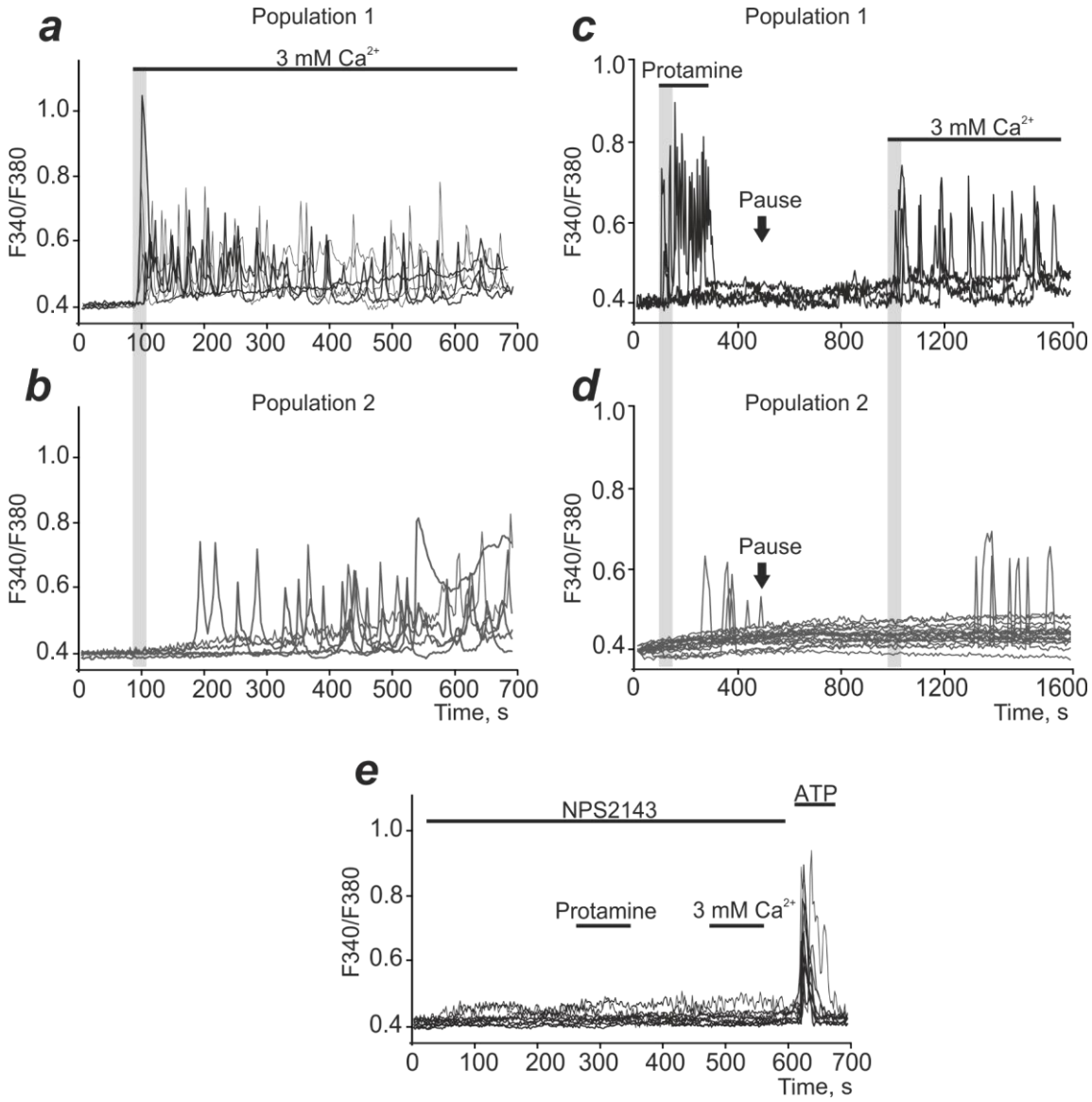

Fig. 1. Activation of the calcium sensitive receptor (CaSR) in differentiated white adipocytes generates Ca^{2+} signals and divides cells into 2 populations. *a, c* – an increase in $[Ca^{2+}]_o$ up to 3 mM (*a*, *n* = 14) or a CaSR activator, protamine (30 μg/ml, *c*, *n* = 8) cause a rapid generation of Ca^{2+} signals in 23 ± 11% of white adipocytes in the microscope field of view. *b, d* – an increase in $[Ca^{2+}]_o$ up to 3 mM (*b*, *n* = 6) or an activator of CaSR, protamine (30 μg/ml, *d*, *n* = 21) in the second population (77 ± 46%) of white adipocytes induces the generation of Ca^{2+} signals with a lag period. *e* – a selective antagonist of CaSR, NPS2143 (3 μM) completely suppresses the Ca^{2+} responses of white adipocytes to an increase in $[Ca^{2+}]_o$ and protamine (30 μg/ml), *n* = 19. Representative Ca^{2+} responses of white adipocytes are presented. All experiment was performed in 4 repetitions (*N* = 4) on three separate cell cultures

imaging data were compared with the immunocytochemical staining image and thus determined to which population each individual adipocyte belongs. It turned out that in the cell culture of white mouse adipocytes, CaSR is expressed on average in 12 ± 9.7% of cells, and it is this population of adipocytes (Fig. 2a, indicated as 1 – 8) that responds with Ca^{2+} signals to increase $[Ca^{2+}]o$ and protamine without lag-period (Fig. 2b – $CaSR^+$).

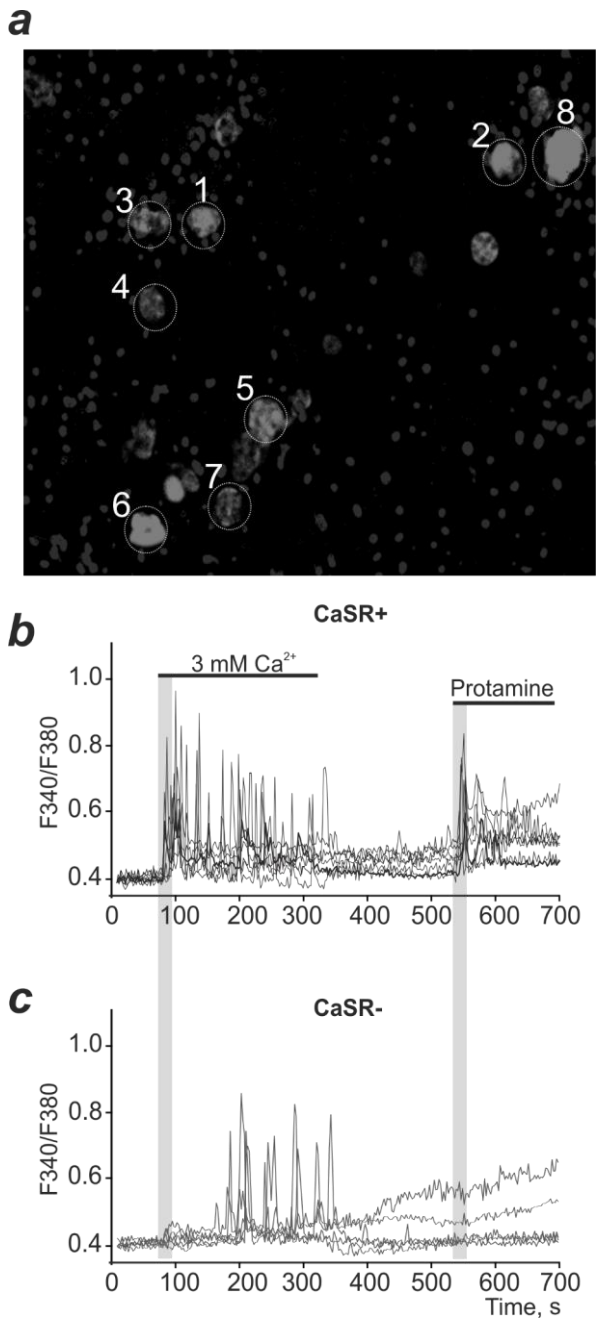

Fig. 2. Immunocytochemical staining of the cell culture of mouse white adipocytes with antibodies against CaSR and their Ca^{2+} responses to an increase in $[Ca^{2+}]_o$ and protamine

a – immunocytochemical staining of cells with antibodies against CaSR. Cells marked as 1–8 express CaSR, while in the remaining cells (nuclei stained with Draq5) fluorescence of anti-CaSR antibodies was not detected.

b, c – Ca^{2+} responses of adipocytes expressing ($CaSR^+$, *b*, $n = 8$) and not expressing CaSR ($CaSR^-$, *c*, $n = 5$). Signals on panel (*b*) correspond to cells marked 1–8 on panel *a*, while panel (*c*) shows typical signals from $CaSR^-$ adipocytes. Representative Ca^{2+} responses of white adipocytes are presented. All experiment was performed in 3 repetitions ($N = 3$) on three separate cell cultures

Whereas in other cells (Fig. 2a, without designations – nuclei, $CaSR^-$), CaSR expression was not detected and Ca^{2+} signals were generated in them after the lag period, after the response of the first adipocyte population.

Thus, activation of CaSR causes the generation of Ca^{2+} signals (mainly oscillations) in the minor population of white adipocytes expressing CaSR. Whereas in most cells, the expression of this receptor was not detected and an increase in $[Ca^{2+}]o$ or a selective activator causes Ca^{2+} signals that occur due to a different mechanism after a lag period of varying duration.

CaSR activation induces ATP secretion by white adipocytes and regulates the expression of lipolysis modulator genes. White adipocytes are not only a depot for triglycerides, but also active secretory cells (Ailhaud, 2006). In addition, using astrocytes as an example, it has been shown that an increase in

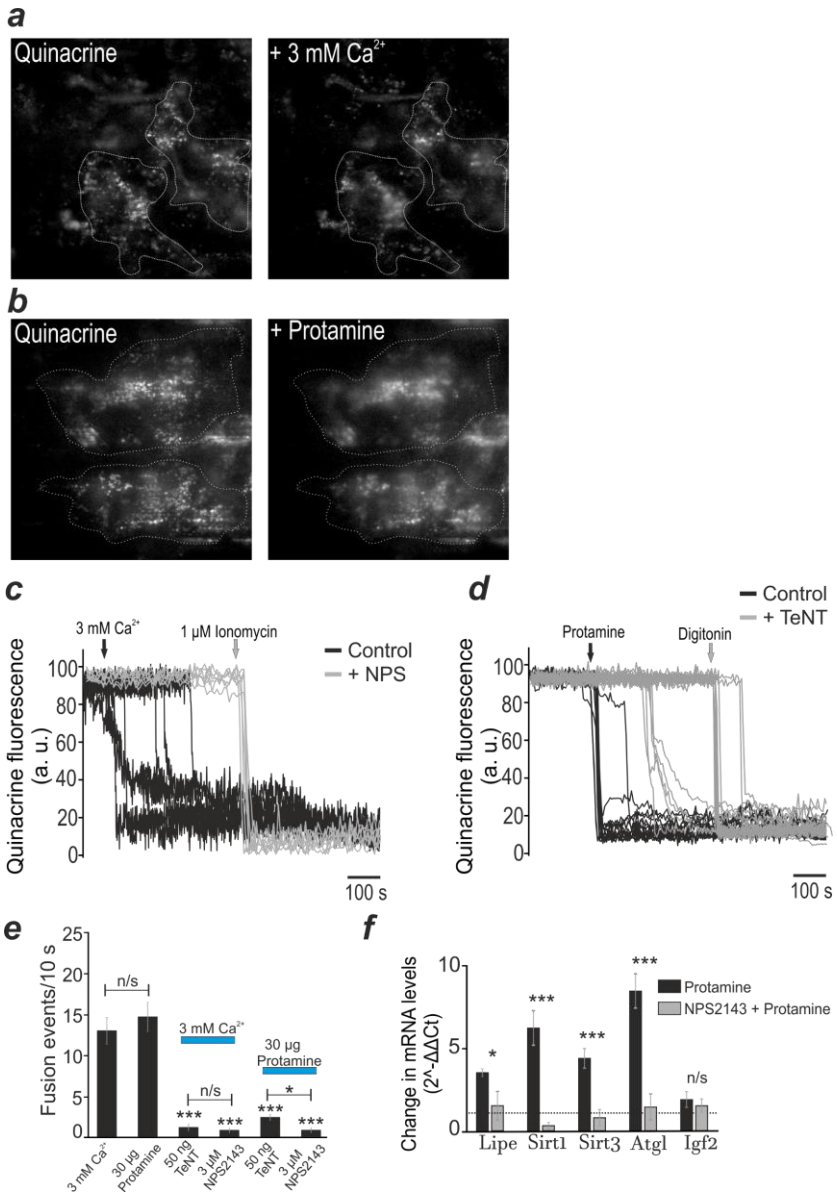

Fig. 3. Secretion of ATP-containing vesicles upon activation of CaSR by increasing $[Ca^{2+}]_o$ up to 3 mM (*a, c*) or by application of 30 μg/ml protamine (*b, d*). Effect of CaSR activation on the expression of lipolysis regulating genes. *a, b* – images of the near-membrane localization of ATP-containing vesicles stained with quinacrine, obtained using TIRF microscopy before and after increasing $[Ca^{2+}]_o$ to 3 mM (*a*) or application of the CaSR agonist – 30 μg/ml protamine (*b*). Two white adipocytes are presented. *c, d* – dynamics of the secretion of ATP-containing vesicles in time, obtained using TIRF microscopy, reflecting the increase in secretion (decrease in quinacrine fluorescence) upon application of an 3 mM Ca^{2+} (*c*) or 30 μg/ml protamine (*d*). Gray curves – ATP secretion against the background of CaSR antagonist – 3 μM NPS2143 (+NPS) and calcium-dependent secretion blocker 50 ng/ml tetanus toxin (+TeNT). At the end of quinacrine fluorescence registration, Ca^{2+} ionophore – ionomycin (1 μM) or detergent – digitonin (1 μg/ml) was added. *e* – The effect of suppressing the secretion of ATP-containing vesicles upon activation of CaSR in white adipocytes using a selective CaSR antagonist – NPS2143 and a calcium-dependent vesicular secretion blocker – tetanus toxin (TeNT). Averaged data from three independent experiments are presented. *f* – Effect of 24-hour incubation of white adipocytes with CaSR activator – protamine (30 μg/ml) and in the presence of a selective CaSR antagonist – NPS2143 (3 μM). The level of gene expression in the control (without influences) is taken as 1 (indicated by the dashed line). The differences between the columns were estimated with paired t-test. * – differences are statistically significant, $p < 0.05$; *** – differences are statistically significant, $p < 0.001$. n/a – differences are not significant ($p > 0.05$)

$[Ca^{2+}]i$ under various influences causes the secretion of gliotransmitters and, first of all, ATP (Gaidin et al., 2019; Bazargani &Attwell 2016). Moreover, the delay in the generation of Ca^{2+} signals by the majority of white adipocytes upon activation of CaSR in a minor cell population may suggest the presence of paracrine activation by ATP.

Using TIRF microscopy and staining of ATP-containing vesicles of white adipocytes with a vital probe quinacrine (Fig. 3), we were able to show that an increase in $[Ca^{2+}]o$ up to 3 mM (Fig. 3a, +3 mM Ca^{2+}) or application of an agonist CaSR-30 μg/ml protamine (Fig. 3b, +Protamine) cause ATP secretion, recorded as a rapid and significant decrease in the fluorescence intensity of quinacrine in the adipocyte (Fig. 3a, d). The level of secretion of ATP-containing vesicles in time upon activation of CaSR by an increase in $[Ca^{2+}]o$ or protamine averages 12±3 and 15±4 vesicles per 10 seconds, and a decrease in quinacrine fluorescence occurs predominantly within 5–10 seconds after stimulation of adipocytes (Fig. 3e). An increase in $[Ca^{2+}]o$ to 3 mM against the background of the CaSR antagonist – 3 μM NPS2143 does not lead to the secretion of ATP-containing vesicles, and only the addition of Ca^{2+} ionophore – 1 μM ionomycin leads to complete fusion of the vesicles with the cell membrane (Fig. 3c, +NPS; Fig. 3e). The fact that the process of secretion of ATP-containing vesicles is Ca2+-dependent is indicated by an experiment in which the application of 30 μg/ml protamine was performed against the background of an inhibitor of Ca2+-dependent secretion – 50 ng/ml tetanus toxin, which almost completely suppressed the secretion of ATP-containing vesicles in response to 3 mM Ca^{2+} (Fig. 3e) and significantly suppressed secretion upon application of protamine (Fig. 3d, +TeNT; Fig. 3e).

Ca2+ ions as a secondary messenger regulate most intracellular processes, including gene expression. To elucidate the role of CaSR activation and vesicular ATP secretion in the physiological effects of white adipose tissue, experiments were carried out on 24-hour incubation of cells with the addition of 30 μg/ml of CaSR activator – protamine (Fig. 3f, black bars) and protamine together with the CaSR antagonist – NPS2143 (3 μM, Fig. 3f, gray bars). After these treatments, total RNA was isolated and gene expression analysis was performed using real-time PCR. After 24-hour exposure to protamine, there is an increase in the level of expression of genes Lipe, Sirt1, Sirt3 and Atgl, encoding hormone-sensitive lipase (HSL), sirtuins 1 and 3, triglyceride lipase by 4.3 times, 7.6, 5.1 and 8 , 6 times, respectively (Fig. 3f). Against the background of NPS2143, a cancellation of the protamine effect is observed and the level of expression of the Lipe, Sirt3, and Atgl genes does not significantly differ from the control, and the expression of Sirt3 even decreases by almost 2 times (Fig. 3f, gray bars). Interestingly, CaSR activation does not affect the expression level of the Igf2 gene encoding insulin-like growth factor-2 (Fig. 3f).

Thus, activation of CaSR in white adipocytes causes calcium-dependent vesicular ATP secretion, which may manifest itself in an increase in the expression level of genes encoding proteins responsible for the activation of lipolysis in white adipocytes.

Discussion

The dynamics of $[Ca^{2+}]i$ regulates key intracellular processes in healthy adipose tissue – differentiation, lipid accumulation, and insulin signaling (Shi *et al.*, 2000; Szabo *et al.*, 2008; Turovsky *et al.*, 2016). At the same time, pathological Ca^{2+} signals activate many processes leading to obesity and other chronic pathological conditions (Guerrero-Hernandez & Verkhratsky, 2014). In white adipocytes, Ca^{2+} ions can act as activators of lipid accumulation through two mechanisms – inhibition of lipolysis through suppression of hormone-sensitive lipase (Xue et al., 2001) and activation of lipogenesis through increased expression and activity of fatty acid synthase (Jones et al., 1996). At the same time, the role of $[Ca^{2+}]i$ as a lipolysis inhibitor is also known and is more complex. For example, calmodulin is involved in the inhibition of lipolysis of white adipocytes through the activation of hormone-activated lipolysis, and is also a central link in lipolysis

during exercise (Kawai, 1985; Izawa & Komabayashi, 1994). It is known that in cultivated human adipocytes, the application of 1 μM calcium mimetic cinacalcet reduces the release of glycerol by 20%, which may indicate the participation of CaSR in the suppression of basal lipolysis (Cifuentes *et al.*, 2012). A CaSR agonist, GdCl3, has a similar effect, which, through an increase in $[Ca^{2+}]i$, changes the activity of lipolysis regulating enzymes (He et al., 2011). Such differences in physiological effects mediated by changes in Ca^{2+} dynamics should depend on the parameters of Ca^{2+} signals. Thus, there is an opinion that the antilipolytic effect of Ca^{2+} ions occurs with a significant and prolonged increase due to cell depolarization (Xue et al., 2001) , while prolipolytic effects are observed as a result of more local Ca2+ events upon activation of G proteins (Kawai, 1985, van Harmelen *et al.*, 2008). In our experiments, when CaSR is activated, long-term high-amplitude Ca^{2+} signals occur, including stable Ca^{2+} oscillations, and as a result, 24-hour exposure to a CaSR agonist leads to an increase in the expression of genes involved in lipolysis inhibition.

In our experiments, the effect on CaSR caused the activation of the minor population ($CaSR^{+}$) of white adipocytes, and an increase in $[Ca^{2+}]i$ in them led to the paracrine activation of the remaining adipocytes through ATP secretion. The calcium-dependent process of ATP secretion by white adipocytes was demonstrated by us using Tirf microscopy. It is known that preincubation of white adipocytes with ATP inhibits insulin-stimulated glucose uptake (Chang & Cuatrecasas, 1974). The activation of P2-purinoreceptors under the action of ATP causes an increase in $[Ca^{2+}]i$ and enhances lipolysis through the activation of protein kinase A, which also correlates with a decrease in the level of leptin production (Lee *et al.*, 2005).

The following enzymes are of key importance in the regulation of lipolysis and lipogenesis of fat cells – hormone-sensitive lipase (Lipe), sirtuins (Sirt), triglyceride lipase (Atgl) and insulin-like growth factor (Igf2) (Armani *et al.*, 2017) and changes in the expression of genes that encode them. may reflect the balance between lipolysis-lipogenesis processes. Activation of CaSR by the activator protamine, after 24 hours of exposure, led to an increase in the expression of all these genes, with the exception of Igf^2. It is known that the activation of hormone-sensitive lipase is necessary for lipolysis caused by the action of hormones, which, in turn, cause the generation of Ca2+ signals in the cytosol of adipocytes (Turovsky *et al.*, 2013; Dolgacheva *et al.*, 2016), similar to the action of increasing $[Ca^{2+}]o$ or CaSR agonist. Moreover, in mice with knockout of hormone-sensitive lipase, abnormalities in the morphology of white adipocytes, mechanisms of lipid accumulation in the form of DAGs, and their differentiation were revealed (Shen et al., 2011; Fortier et al., 2004). Triglyceride lipase is also involved in the process of lipolysis of white adipocytes and regulates precisely its initial stage – cleavage of TAG into DAG (Schweiger *et al.*, 2006) and the level of Atgl gene expression increased along with Lipe. In addition to the above lipases, sirtuins, which are associated with cell survival, apoptosis, inflammation, glucose and lipid homeostasis, play an important role in the physiology of white adipose tissue (Sanders *et al.*, 2010). In our experiments, there is an increase in the expression of Sirt1 upon activation of CaSR, which, on the one hand, can prevent the accumulation of lipid inclusions, and, on the other hand, can promote the differentiation of adipocytes. Sirt1 activation also promotes the phosphorylation of AMPK and inhibits the synthesis of fatty acids and an increase in the number of lipid inclusions in fat cells in response to high glucose levels (Hou *et al.*, 2008). Sirt3 is expressed in mitochondria and is involved in the regulation of the activity of mitochondrial enzymes responsible for glycolysis, fatty acid oxidation, and amino acid catabolism. Sirt3 is required for the activation of the bioenergetic functions of mitochondria in the early stages of adipocyte differentiation (Parihar *et al.*, 2015). At the same time, the level of expression of the studied genes when exposed to protamine against the background of the selective CaSR antagonist NPS2143 is either at the control level or, in the case of Sirt1, decreases, which indicates that the antilipolytic effect of CaSR

activation is canceled. In addition, as shown in this study, against the background of NPS2143, the secretion of ATP-containing vesicles is inhibited and, therefore, the paracrine activation of the network of white adipocytes, which correlates with the cancellation of the effect of 24-hour incubation of cells with a CaSR agonist.

In this work, we have shown that CaSR is expressed in a small population of white adipocytes, however, an increase in $[Ca^{2+}]o$ or a selective activator of this receptor leads to Ca2+-dependent secretion of ATP-containing vysicles, which causes the activation of all neighboring white adipocytes (which do not express CaSR) and the generation of short-term and stable vibrational Ca^{2+} signals. An increase in $[Ca^{2+}]i$ upon activation of CaSR correlates with an increase in the level of expression of genes encoding proteins responsible for triglyceride lipolysis, and a selective CaSR antagonist cancels this effect of lipolysis activation.

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