Chapter 11

Feedback Control of Second Messengers Signaling Systems in White Adipose Tissue Adipocytes in Healthy State and Its Loss at Adiposity

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Abstract

Second messengers Ca^{2+}, IP3, cAMP, NO, cGMP, and cADP ribose are incorporated as obligatory elements into multivariable Ca^{2+}-signaling system, which integrates incoming signals of hormones and neurotransmitters in white adipocytes. This cross-controlled system includes two robust generators (RGs) of rhythmic processes, involving phospholipase C- and NO-synthase-dependent signaling networks (PLC-RG and NOS-RG). Multi-loop positive feedback control of both RGs provides their robustness, multistability, signaling interplay, and extreme sensitivity to the alterations of incoming signals of acetylcholine, norepinephrine, insulin, cholecystokinin, atrial natriuretic peptide, bradykinin, and so on. Hypertrophy of cultured adipocytes and of mature cells, isolated from epididymal white adipose tissue (eWAT), results in the loss of rhythmicity and development of general hormonal signaling resistance. Preadipocytes isolated from eWAT of obese mice cannot grow and accumulate lipids in the media devoid of fatty acids. However, even low concentrations of palmitoylcarnitine in the media (1 μM) may result in drastic suppression of mRNA expressions of the proteins of Ca^{2+}-signaling system, especially of NOS-RG. Similar alterations of gene expression are observed in eWAT and liver at adiposity. All this may indicate on universal background pathogenic mechanisms. Treatment modalities, which may help to restore deregulation of Ca^{2+}-signaling system and corresponding tissues dysfunction, are discussed briefly.

Keywords: adiposity, adipocyte dysfunction, second messengers, NO, PKG, feedback and cross-control, loss of rhythmicity

1. Introduction

Adipose tissue dysfunction ("adiposopathy") is considered as one of the primary drivers of multifactorial pathological process, ranging from systemic insulin resistance and hypertension to cardiovascular diseases, liver and pancreas dysfunction, and type 2 diabetes (T2D) [1, 2]. Obviously, observed gradual dysfunction of various tissues at T2D is due to the deregulation of metabolic and signaling systems providing the fulfillment of these functions. All these processes of deregulation, especially of signaling systems, may have some universal features, which are being based on the loss of feedback control mechanisms in the systems studied. The identification of these control mechanisms, including crosstalk of signaling pathways, may create new opportunities to identify real targets and develop new options of various diseases treatment.

"Adiposopathy" is characterized by: lipid metabolism deregulation, development of oxidative stress and mitochondrial dysfunction, death of hypertrophied adipocytes, tissue remodeling, loss of fatty acids buffering, and endocrine and immune functions [2–6]. However, the existing data on external hormonal and autonomous feedback control of white adipose tissue (WAT) lipid metabolism (triglycerides—fatty acids turnover) are insufficient to answer the question on the mechanisms providing uncontrolled hypertrophy of adipocytes. Later, based on own results obtained in animal experiments and known literature data, we will try to represent (at first level of approximation) the structures and mechanisms of autoregulation of second messengers signaling systems, which might be functioning in the adipocytes and other types of nonexcitable cells.

2. Calcium, cAMP, and cGMP-related signaling systems, operating in adipocytes of healthy animals

2.1. Brief survey of existing models of adipocyte triglyceride metabolism control

It is known that, acting via lipid kinase (PI3K)/PKB-signaling pathway, insulin may stimulate adipogenesis and triglyceride (TAG) synthesis, by phosphorylating rate-limiting enzymes Acyl-CoA: glycerol-3-phosphate acyltransferases (GPAT1, 4) and phosphatidic acid phosphatase (Lipin) [7, 8]. On the contrary, norepinephrine (NE) promotes dephosphorylation of lipin [8]. Modern viewpoints on the control of TAG hydrolysis to free fatty acids (FFA) are mainly focused on the regulation (phosphorylation) of hormone sensitive lipase (HSL), adipocyte triglyceride lipase (ATGL), and perilipin by PKA and are being based on opposite influence of NE (β-adrenoreceptors; *β-AR*) and of insulin on cAMP concentration and PKA activity [9–14]. Supposed mechanisms of antilipolytic action of insulin include the activation of cAMP phosphodiesterases PDE3,4 and inhibition of PKA activity through Insulin/PI3K/PKB-pathway. In addition to insulin, antilipolytic action may be provided through G-protein-coupled receptors by NE (*α2-AR*), adenosine, prostaglandins, neuropeptide Y, and so on [11–14].

Phosphorylation of key lipases and perilipin by PKG1 is considered as a separate mechanism, involved in the activation of TAG hydrolysis [10, 13, 14]. This signaling pathway, which is

based on the activation of PKG1 via atrial natriuretic peptide receptor A (NPR-A/mGC/cGMP/PKG1-pathway), does not involve nitric oxide (NO) and soluble guanylate cyclase (sGC).

This widely admitted model of TAG-FFA turnover (futile cycle) control describes the regulation of lipid metabolism as external hormonal adjustment, realized through PI3K/PKB, cAMP/PKA, and cGMP/PKG1, that is, as a model devoid of self-control and crosstalk of functioning second messenger signaling systems. Moreover, NO and calcium are not included into consideration as possible messengers, involved in the control of WAT metabolism. Though the results of the last decade indicate that the activation of endothelial NO-synthase (eNOS), NO bioavailability, and recruitment of eNOS/NO/sGC/cGMP/PKG1-signaling chain may protect against obesity, by influencing differentiation and mitochondrial biogenesis in brown fat cells, adipogenesis and lipolysis in white cells, and so on. [15–20]. Besides that, controversial results on the role of Ca^{2+} and calcium-sensing receptors in the regulation of body fat depots [21–23] point on the important role of Ca^{2+} in the mechanisms of self-control of second messengers signaling systems.

2.2. Two Ca^{2+}-dependent signaling systems and rhythmic processes in adipocytes of WAT

Like most of other nonexcitable types of cells, adipocytes possess two types of intracellular Ca^{2+} release channels, located on the membrane of endoplasmatic reticulum: inositpl-1,4,5-triphophate (IP3) receptors (IP3R) and ryanodine receptors (RyR). Both types of receptors are controlled by numerous signaling molecules, including PKA PKG, Ca^{2+}-dependent kinases, various isoforms of PKC, and so on [24–26]. This versatility of control defines the shaping of intracellular Ca^{2+} dynamics, which plays a primary role in the regulation of numerous cellular processes [24]. Ubiquitous oscillations of intracellular Ca^{2+} concentration, which are observed in most of nonexcitable cells [27–30], are often considered as the basic dynamic mechanisms involved in the control of cellular metabolic processes [27–29]. However, the role of Ca^{2+} oscillations and of triggering phenomena is not understood and evaluated yet properly [27, 30]. However, the analysis of such dynamic processes may be very instrumental for the determination and evaluation of operating feedback mechanisms.

Both types of Ca^{2+} release channels possess a fundamental property, called Ca^{2+}-induced Ca^{2+} release (CICR), which may provide Ca^{2+}-sparks, fast oscillations, and spatial waves [24–26]. Gaiting of IP3R is reinforced by IP3, which facilitates binding of Ca^{2+} and channel opening [24, 25]. In other words, Ca^{2+} and IP3 represent crosscoupled messengers targeted to IP3R [31].

As for RyR, according to generally accepted point of view, the regulation of this receptor lacks this kind of symmetry. Cyclic ADP-ribose (cADPr), which is formed from NAD by ADP-ribosyl cyclase (ARC) or ectoenzyme CD38, is not considered as an obligatory coagonist of RyR [26], in spite of existing data on its modulatory role in RyR-channels gaiting and CIRC control [32–37]. Really, in striated muscles RyR-channels gaiting and CIRC are determined mainly by plasmalemmal membrane depolarization [26]. In nonexcitable cells, the primary role in Ca^{2+} homeostasis is supposed to be realized via IP3R [24, 27–30], while modulatory role is delegated to RyR, which may amplify Ca^{2+}-signals produced by IP3-dependent CICR [24–26].

2.2.1. Ca^{2+}/phospholipase C/IP3/IP3R/Ca^{2+} positive feedback signaling system

Numerous external signals, by stimulating Gq proteins and tyrosine kinase (TK) coupled receptors, result in the formation of IP3 by various isoforms of phospholipase C (PLC) [24, 25, 38] with subsequent activation of IP3R-channels and rise of Ca^{2+}in the cytoplasm via CICR mechanism:

$$TK, G_{\alpha q} \rightarrow PLC \rightarrow IP_3 \rightarrow IP_3R \rightarrow Ca^{2+} \quad (1)$$

Realization of IP3-dependent CICR represents short positive feedback loop (PFL) in the system:

$$Ca^{2+} \rightarrow IP_3R \rightarrow Ca^{2+} \quad (2)$$

Being activated by Ca^{2+}, Ca^{2+}-dependent isoforms of PLC may provide functioning of long PFLs [31]:

$$Ca^{2+} \rightarrow PLC \rightarrow IP_3 \rightarrow IP_3R \rightarrow Ca^{2+} \quad (3)$$

Therefore, IP3R-dependent Ca^{2+}–signaling system represents two loops' generator (**Figure 1**), in which short PFL (shown as broken arrow 1) is embedded by long PFL (arrow 2). This duplicating loop may provide the robustness with respect to the alteration of systems parameters [39, 40]. Released by IP3R-channel intracellular Ca^{2+} may provoke RyR-dependent CICR, which, in turn, might further amplify initial signals and support generation of Ca^{2+} oscillations and/or wave propagation. Inhibition of IP3R, due to phosphorylation of IP3R by Ca^{2+}-activated CaM-kinases II (CaMKII), represents stabilizing negative feedback loop (NFL) (dotted line 4 with sign T).

This Ca^{2+}/PLC/IP3/IP3R/Ca^{2+}-robust generator (PLC-RG) is cross-activated by adenylate cyclase (AC)/cAMP/PKA-signaling pathway, owing to phosphorylating of IP3R (and RyR) by PKA (arrows 7, 8). Inhibition of AC and activation of PDE3, produced by the phosphorylation of both enzymes by PKA [42], may provide the functioning of two stabilizing NFLs in this pathway (dotted line 5 and arrow 6). And finally, PI3K/PKB/NO/cGMP/PKG1-signaling pathway participates in negative crosstalk with both systems, by inhibiting IP3R via PKB (dotted line 10) and PKG1 (dotted line 12) and by activating PDE3 through PKB (arrow 9) [10, 11]. Well-known inhibition of PDE3B by cGMP (dotted line 11) [9] contradicts this logic of system's self-control and, apparently, may be realized at high concentrations of cGMP.

Cross-inhibition of eNOS, based on its phosphorylation by PKC [41], is shown at the bottom of **Figure 1** (dotted line 14). Cross-inhibition of PLCβ activity, which may be realized with the involvement of PKG1 and PKA [42], is omitted for the simplicity.

It ought to outline that, besides combined action of IP3 and Ca^{2+} on IP3R, PLC-RG has second point of regulatory symmetry. The entry into the system through PLC is carried out by combined activation of PLC by $G_{\alpha q}$-proteins (or TKs) and Ca^{2+} (**Figure 1**).

PLC-RG represents a highly nonlinear dynamic system, which incorporates the family of two nested PFLs. Due to that, this system possesses the properties of multistable generator, which

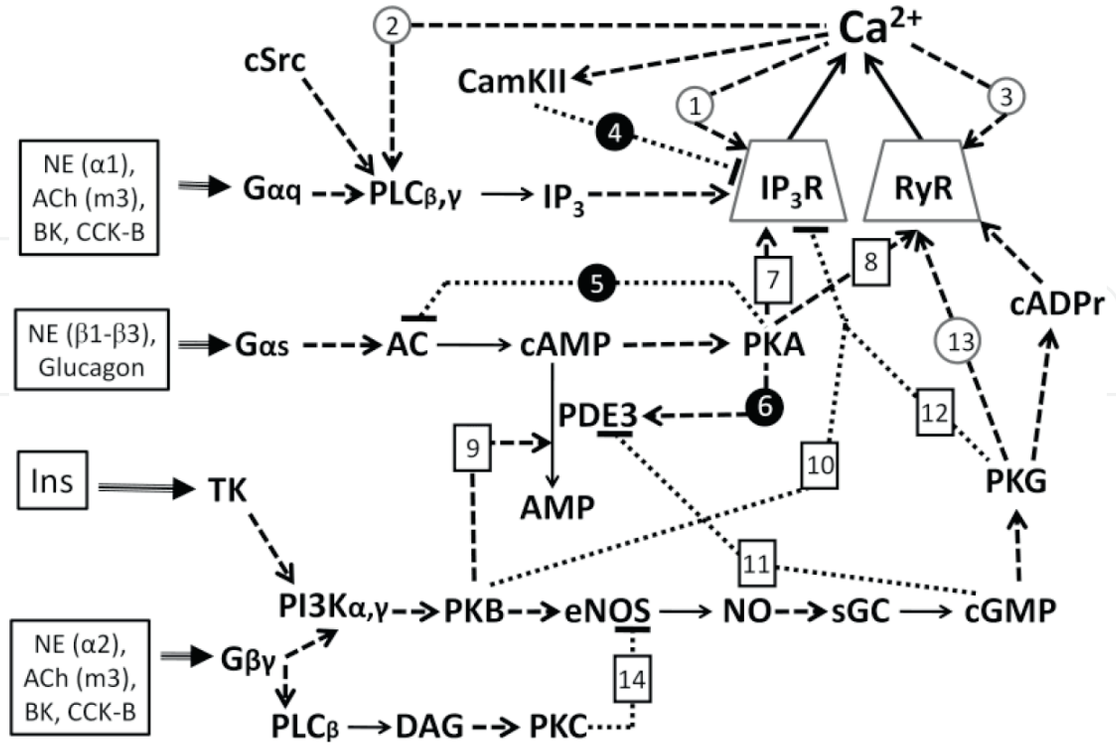

Figure 1. PLC/IP3/IP3R/Ca^{2+}-signaling system and its cross-control by AC/cAMP/PKA and PI3K/PKB/NO/cGMP/PKG-signaling pathways. All abbreviations and explanations are given in the text. Various types of activation and inhibition (direct regulations or covalent modifications) are indicated as broken arrows and dotted lines (with symbol T), correspondingly. Positive and negative feedback loops are marked with white or black circles and have the numbers 1–3 and 4–6, correspondingly. Crosstalk loops are marked by the squares and have the numbers 8–12 and 14. Various hormones and neurotransmitters (with corresponding receptors), activating G-proteins and TKs, are placed in the boxes.

may produce: steady state regimes with different concentrations of intracellular Ca^{2+} (and of other second messengers), triggering phenomena, Ca^{2+} spikes, ordinary and complex (multiperiodic or chaotic) oscillations, and waves propagation. Realization of described regimes depends on the system's parameter values, that is, on the set of enzyme and channel activities and agonist affinities. All such regimes were observed experimentally in various types of cells [43–51] and were reproduced in mathematical models [52–54]. Apparently, all ranges of these dynamic regimes were observed for the first time on isolated hepatocytes [45]. In most of the published experiments registered Ca^{2+} oscillations and waves were attributed to the functioning of PLC-RG, including regimes elicited by NE [45], Ach [47, 49], histamine [44], glutamate [48], and so on.

In cultured adipocytes of epididymal WAT, periodic Ca^{2+} signals and spikes, which depend on PLC-RG activity, may be evoked by fetal bovine serum [50, 51], NE [55–57], Angiotensin II (Ang II) [58, 60], cholecystokinin (CCK) and ANP [58], insulin [59], and bradykinin (BK) (Turovsky et al., submitted for publication).

2.2.2. Ca^{2+}/eNOS/NO/sGC/cGMP/PKG/ARC/cADPr/RyR/ Ca^{2+} positive feedback signaling system

In contrast to all abovementioned agonists, ACh may elicit Ca^{2+} oscillations in WAT adipocytes (9DIV) by involving another mechanism, which does not implicate PLC and IP3R [61].

The effect of ACh is realized via *m3*-muscarinic receptors (*m3-MR*) and Gβγ subunits of corresponding Gq-proteins. This kind of rhythmic activity is characterized by Ca^{2+} and NO oscillations with phase shift about 180°. Remarkably, insulin, Ang II, CCK, and BK may also evoke Ca^{2+} and NO oscillations by activating second oscillatory mechanism [59, 60].

Earlier works, performed by several groups, depicted that NO, cGMP, and cADPr may induce Ca^{2+} mobilization and oscillations in hepatocytes [64, 65], smooth muscle cells [66, 67], and T-cells [68], involving RyR. The model, proposed for first time to explain mobilization of intracellular Ca^{2+} via NO/cADPr-dependent signaling pathway [33, 34], was based on known phosphorylation and activation of ARC by PKG1 [36, 37] and on the facilitation of RyR-channels gaiting by newly formed cADPr [32, 33]. This model include following signaling chain:

$$\text{eNOS} \rightarrow \text{NO} \rightarrow \text{sGC} \rightarrow \text{cGMP} \rightarrow \text{PKG1} \rightarrow \text{ARC} \rightarrow \text{cADPr} \rightarrow \text{RyR} \rightarrow Ca^{2+} \quad (4)$$

Well-known activation of eNOS by Ca^{2+} [69–71] transforms this linear chain into long PFL, which creates basic loop of NOS-dependent robust generator (NOS-RG):

$$\begin{aligned} Ca^{2+} &\rightarrow \text{eNOS} \rightarrow \text{NO} \rightarrow \text{sGC} \rightarrow \text{cGMP} \\ &\rightarrow \text{PKG1} \rightarrow \text{ARC} \rightarrow \text{cADPR} \rightarrow \text{RyR} \rightarrow Ca^{2+} \end{aligned} \quad (5)$$

Speaking on the math language, formation of long PFL(5) represents necessary conditions for the functioning of NOS-RG. PFL(5) is very sensitive to any input in it. The application of ANP (input of cGMP), 8-br-cGMP, SNAP (influx of NO), NAD (substrate in the synthesis of cADPr) [61], or activation of Ca^{2+}-influx (by low concentrations of arachidonic acids via store-independent Orai channels) [63], may bring oscillations and triggering regimes in adipocytes.

ACh and all abovementioned hormones, activating TK or/and G-protein-coupled receptors, provide sufficient conditions for stable functioning of NOS-RG, activating eNOS via axis:

$$\text{TK}, G\beta\gamma \rightarrow \text{PI3K}\gamma \rightarrow \text{PKB} \rightarrow \text{eNOS} \quad (6)$$

Incubation of cultured adipocytes with the inhibitors of the proteins of this axis prevents the activation of NOS-RG by ACh, insulin, CCK, and Ang II [61–63].

Thereby, NOS-RG also has both kinds of symmetries, including: (a) activation of RyR by cross-coupled second messengers Ca^{2+} and cADPr and (b) combined activation of eNOS by Ca^{2+} and PKB (via axis (6)).

The model of self-control of NOS-RG is presented in **Figure 2**. Besides short and long PFLs, based on cADPr-dependent CICR and activation of eNOS by Ca^{2+} [69–71] (broken arrows 1, 2), this model incorporates six PFLs (arrows 3–8) and three NFLs (dotted lines 10, 12, and broken arrow 11). Group of PFLs (arrows 3–6), based on the phosphorylation and activation of eNOS [70, 71] and of PKB by CaMKIV and AMPK [72, 73], provide the amplification of basic PFLs and robustness of NOS-RG.

PKG1 occupies a central position in the autoregulation of NOS-RG. Feedforward activation of RyR [26] and feedback activation of eNOS [74–76] and PKB [72, 77] by PKG1 (arrows 7–9)

raise the reliability of this system. Feedback inhibition of sGC [78] and activation of PDEV [79] by PKG1 are directed to lower the level of cGMP in NOS-RG (line 12 and arrow 11). The inhibition of external Ca^{2+} influx, realized via inhibition of TRP channels by PKG1 [80–82] (line 10), may reinforce these NFLs.

The reliability and low sensitivity to noise and parameters alterations of technical systems is primarily attained by multiple negative feedback control and duplication of operating elements [39, 40, 83, 84]. PFLs, in contrast to NFLs, may enhance system's sensitivity to changes of internal parameters and noise by amplifying incoming signals.

Basic structures of autoregulation of PLC-RG and NOS-RG involve the families of nested PFLs. Such unusual structures may create new properties of analyzed system: combination of extreme sensitivity to the alterations of incoming signals and the reliability, provided by the redundancy of PFLs. Due to that, both systems may be considered as robust but sensitive integrators of multiple hormonal signals.

Positive cross-control of NOS-RG, fulfilled by AC/cAMP/PKA-signaling pathway, is indicated by broken arrows 13–15 in **Figure 2**. This control is directed to internal elements of main PFLs (arrows 5, 6), being addressed to RyR, ARC, and PKB. Owing to that, NOS-RG might have high sensitivity to this kind of cross-control. Corresponding examples will be

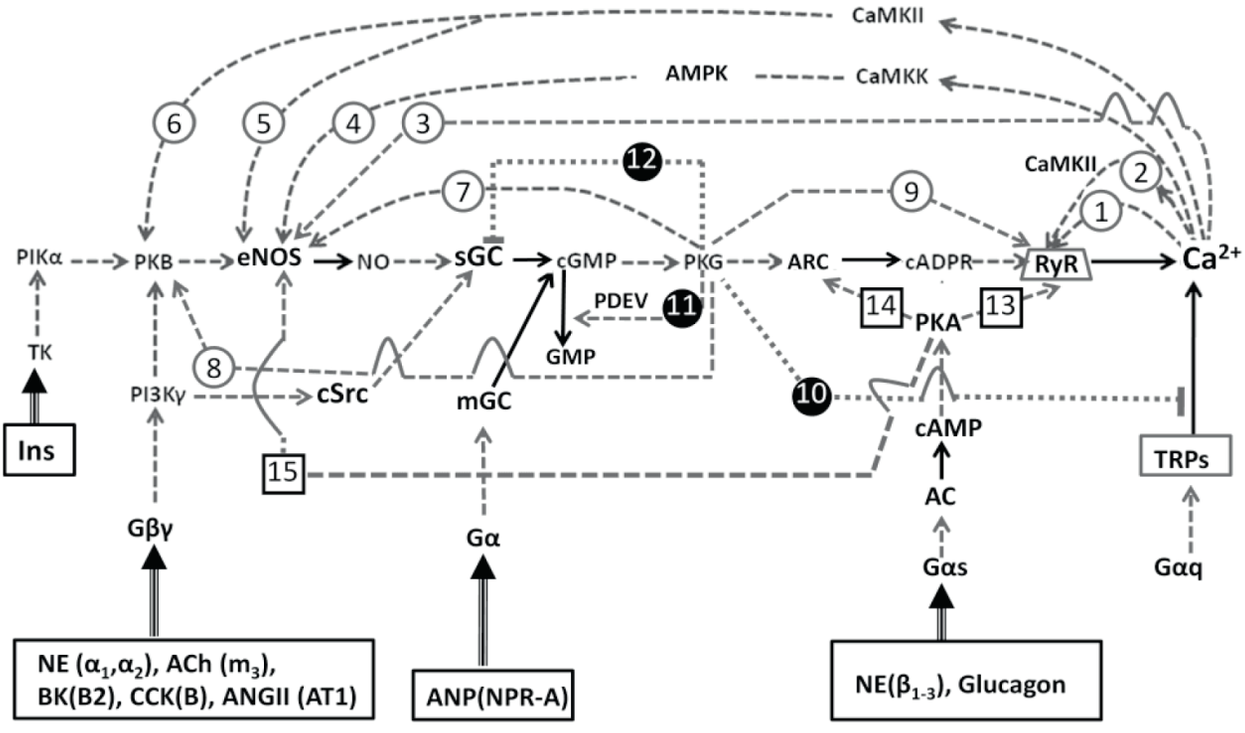

Figure 2. PI3K/PKB/eNOS/NO/sGC/cGMP/PKG1/ARC/cADPr/RyR/Ca^{2+}-signaling system with its system of autoregulation and cross-control by AC/cAMP/PKA-signaling pathways. All abbreviations and explanations are given in the text. Various types of activation and inhibition are indicated as broken arrows and dotted lines (with symbol T), correspondingly. The family of nested positive feedback loops (arrows with white circles) has the numbers from 1 to 8. Positive feedforward loop is numbered as 9. Negative feedbacks (marked by black circles) have the numbers 10 through 12. Crosstalk loops, describing positive impact of AC/cAMP/PKA-signaling pathway, have the numbers 13 through 15. Various hormones and neurotransmitters (with corresponding receptors), activating G-proteins and TKs, are placed in the boxes.

presented later. However, AC/cAMP/PKA-signaling pathway is under negative cross-control of NOS-RG (**Figure 1**). Robustness of NOS-RG and complexity of its crosstalk with AC/cAMP/PKA-signaling pathway represent serious problems in experimental studies and mathematical modeling of such systems.

2.3. Oscillatory and triggering regimes registered in adipocytes

Table 1 summarizes our earlier results, characterizing action of several hormones and neurotransmitters on Ca^{2+}-signaling systems of cultured epididymal adipocytes (9DIV) of white healthy mice. ACh, activating *m3*-muscarinic receptors (*m3-MR*), may elicit periodic Ca^{2+} oscillations in 70–80% of the cells. About 10–15% of the cells respond by spikes. Rest of the cells is silent. Applied inhibitory analysis indicates the implication of NOS-RG [61]. In contrast to Ach, NE by activating PLC-RG via *α1A-AR* evokes Ca^{2+} oscillations in 30–40% of the cells. Subsequent application of NE, after washing of cultured cells of Ach, may induce Ca^{2+} oscillations in the same percentage of cells. Fast monomodal or complex multimodal oscillations may be observed in dependence of cell size [57]. Two lines of numbers at the fifth row describe these two limits of oscillations periods, which were registered in cultures studied.

In comparison with ACh, ANP, and NE, peptide hormones insulin, Ang II, and BK (**Table 1**) may evoke rhythmic activity by involving first or second oscillatory mechanisms (PLC-RG or NOS-RG) in dependence of cellular culture used. Besides that, insulin, CCK, BK, and ANP may often elicit complex multiperiodic and chaotic Ca^{2+} oscillations.

Agonists	**ACh [61]**	**NE [51]**	**Ins [59]**	**Ang II [60]**	**ANP [58, 61]**	**CCK-4 [58]**	**BK** **
Receptors involved and concentrations of agonists used	m3	α1	TK	AT-1	NPR-A	CCK-B	B2R
	1-5 μM	1-5 μM	3-5 nM	300-500 nM	1-5 μM	1-10 nM	0.3-10 μM
PLC-RG, % of cells with rhythmic activity	—	30–40	20–30	20–30	—	20–30	30–40
NOS-RG, % of cells with rhythmic activity	70–80	—	15–25	30–35	30–40	20–40	25–30
Periods of oscillations (s) *	5–60	20–75	20–30	20–50	20–50	25–30	10–30
	100–300	100–300	50–150	75–200	200–300	300–500	200–500

In the table, second and third rows describe, which of two Ca^{2+} signaling systems is turned on by corresponding agonist. Numbers, presented at these rows, show average percentage of all cells in the cultures tested, which generate mono and multimodal oscillatory regimes, or chaotic oscillations. *Periods of minimal and maximal modes of oscillations, observed in the cells of different size, are presented in fourth row. In each experiment 5–10% of all cells were nonresponsive. Rest of the cells was characterized by Ca^{2+} spikes. From 10 to 15 experiments were used for each agonist applied. The number of monitored cells in each culture was 80–100 cells. References are indicated in the square brackets in top row. **Taken from: Turovsky et al., submitted to publisher.

Table 1. Involvement of two Ca^{2+}-signaling systems: PLC and eNOS-dependent robust generators (PLC-RG and NOS-RG) in rhythmic processes evoked by hormones and neurotransmitters in cultured epididymal adipocytes (9DIV) of white 4–6 weeks old male mice.

2.4. Some elements of the control of both Ca^{2+}-signaling systems

2.4.1. Control of IP3R by PKG1

Preliminary results, obtained with fluorescent antibodies staining, indicate smooth dense distribution of IP3R in adipocytes in comparison with smooth but thin distribution of RyR (Turovsky et al., submitted to publisher). This difference corresponds to substantial difference in mRNA expression of the subtypes of IP3R and RyR-receptor proteins (see below). Due to the expression of both types of Ca^{2+}-channels in adipocytes, we might expect their tandem operation under the action of ACh. However, preincubation of cultured cell with PLC or IP3R inhibitors does not alter ACh effects [61]. Moreover, combined application of PLC inhibitors and IP3R antagonists added after ACh may only diminish the amplitude of Ca^{2+} oscillations by10–15% [61]. All this may indicate that expected tandem operation of RyR and IP3R, or supportive role of IP3R [24, 26], is not realized due to inhibitory action of PKG1 on IP3R (**Figure 1**, dotted line 12) and, possibly, on PLCβ. Recent data, demonstrating endothelium-dependent suppression of AVP-evoked Ca^{2+} oscillations in microvessel's myocytes by ACh, support this conclusion [85]. Taken together, these results may indicate universal role of the control of IP3R by PKG1. To stress the question, we might also speculate that, in spite of low protein content of NOS-RG in adipocytes, high activity of this system is supported (reinforced) by unusual multiloop feedback control.

2.4.2. Robustness of NOS-RG: Impact of PFLs, based on activation of several targets by CaMKII and AMPK

CaMKII may be involved in the activation of RyR, eNOS, and PKB (arrows 3, 5, 6 at **Figure 1**). AMPK, being activated by Ca^{2+}-dependent CaMKIV, may also promote further activation of eNOS (arrows 4 at **Figure 1**). To break corresponding PFLs, we applied the inhibitors of both enzymes. To avoid nonspecific effects, we used low concentrations of the inhibitors, equal to their *Kd*. Our preliminary studies have shown that the applications of KN-63 (inhibitor of CaMKII) and of Compound C (inhibitor of AMPK) altered the shape of Ca^{2+}-oscillations and even suppressed rhythmic activity in part of the cells (**Figure 3**). Combined effect of both inhibitors was statistically significant ($p \leq 0.02$). Rather weak effect of Compound C on NOS-RG might be explained by the fact that the activation of AMPK by CaMKIV (at the conditions of our experiments) is insufficient to keep required gain of PFL(4) (arrow 4 at **Figure 2**). Besides CaMKIV, the activity of AMPK is controlled by AMP, sirtuins (SIRT1), liver kinase B1(LKB1), and several other kinases [86].

2.4.3. On the involvement of α1,2-AR and β1: 3- AR in the activation of PLC-RG and NOS-RG

Agonist of *α1-AR* phenylephrine (5–10 μM), like to NE (**Table 1**), evokes Ca^{2+} oscillations in 25–30% of the cells, implicating PLC-RG. Sustainability of these oscillations depends on store-operated Ca^{2+} entry into the cells [63].

Agonists of *α2-AR* guanabenz (10 μM) and L-arginine (5–10 mM) may elicit Ca^{2+} spikes in 35–50% of cultured cells, involving NOS-RG [55].

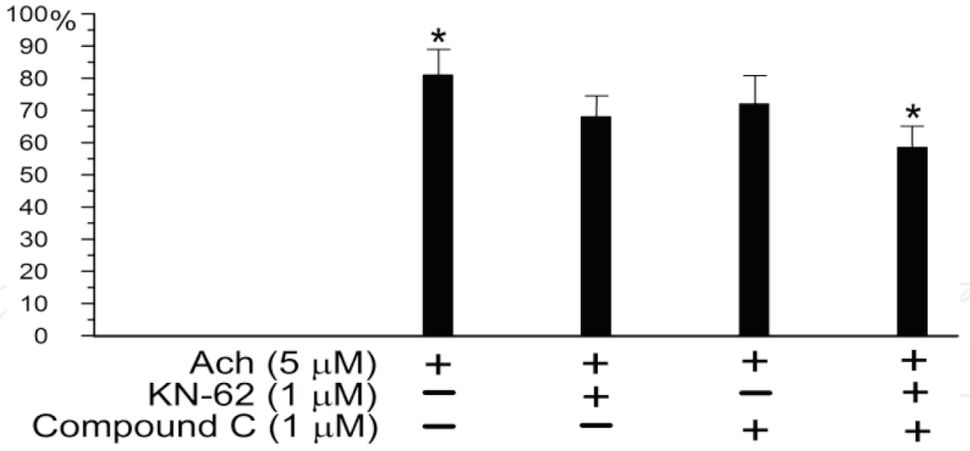

Figure 3. Robustness of NOS-RG. Impact of CaMKII and AMPK on Ca^{2+}- oscillations elicited by ACh in cultured adipocytes (9DIV). Bars represent average number of the cells, which respond to added ACh and the inhibitors of CaMKII (KN-63) and of AMPK (compound C). The inhibitors were added 10 min after the application of ACh. Details are given in the text. N = 6. Data presented as mean ± SD. *denotes statistically significant difference $p \leq 0.02$. Results are taken from: Turovsky et al., submitted to publisher.

Activation of *β1–3- AR* by isoproterenol (3–5 μM) is characterized by slow rise of Ca^{2+} in 40–50% of cells. The antagonists of IP3R and RyR suppress Ca^{2+} responses in 75–80% and 15–20% of activated cells, respectively [62]. Observed difference in this suppression may characterize the impact of PKA on the phosphorylation and activation of IP3R and RyR.

2.4.4. Signaling interplay and sensitivity: Synergistic action of low concentrations of ACh and NE

Low concentrations of ACh, NE, phenylephrine, and L-arginine cannot induce Ca^{2+} responses in adipocytes. However, sequential or combined application of ACh and these agonists display synergistic effects, promoting diverse oscillatory regimes (**Figure 4A–C**) and triggering oscillatory transitions from one stable steady state with low Ca^{2+} level to the second steady state with high Ca^{2+} concentration in the cell (**Figure 4B**, record 2) (Turovsky et al., in publication). This kind of synergy may be explained by combined action of various Gβγ- proteins on signaling axis (6): Gβγ → PI3Kγ → PKB → eNOS.

Combined action of low concentration of ACh and of isoproterenol may elicit complex oscillations (**Figure 4D**), triggering transition from one stable state to another (**Figure 4E**) and Hopf bifurcation, that is, transition from stable steady state to stable oscillatory regime (**Figure 4F**). The mechanism of synergistic action of ACh (*m3-MR*) and of isoproterenol (*β1–3 - AR*) may be based on the activation of axis (6) by ACh, reinforced by the activation of PKB (in this axis) and of ARC and RyR (in NOS-RG) by PKA (arrows 13–15 at **Figure 2**).

2.4.5. cADPr and RyR may play a supportive role in the operation of IP3R and PLC-RG

Nicotinamide (NAM), product and inhibitor of cADPr synthesis by ARC (or CD38), has some influence on Ca^{2+} oscillations evoked by NE. Added NAM (10 mM) may change the shape of oscillations in 20–30% of oscillating cells and suppress rhythmic activity in 15–20% of cells (Turovsky et al., submitted to publisher). This may indicate tandem operation of IP3R and RyR in some part of the cells, having rhythmicity evoked by NE.

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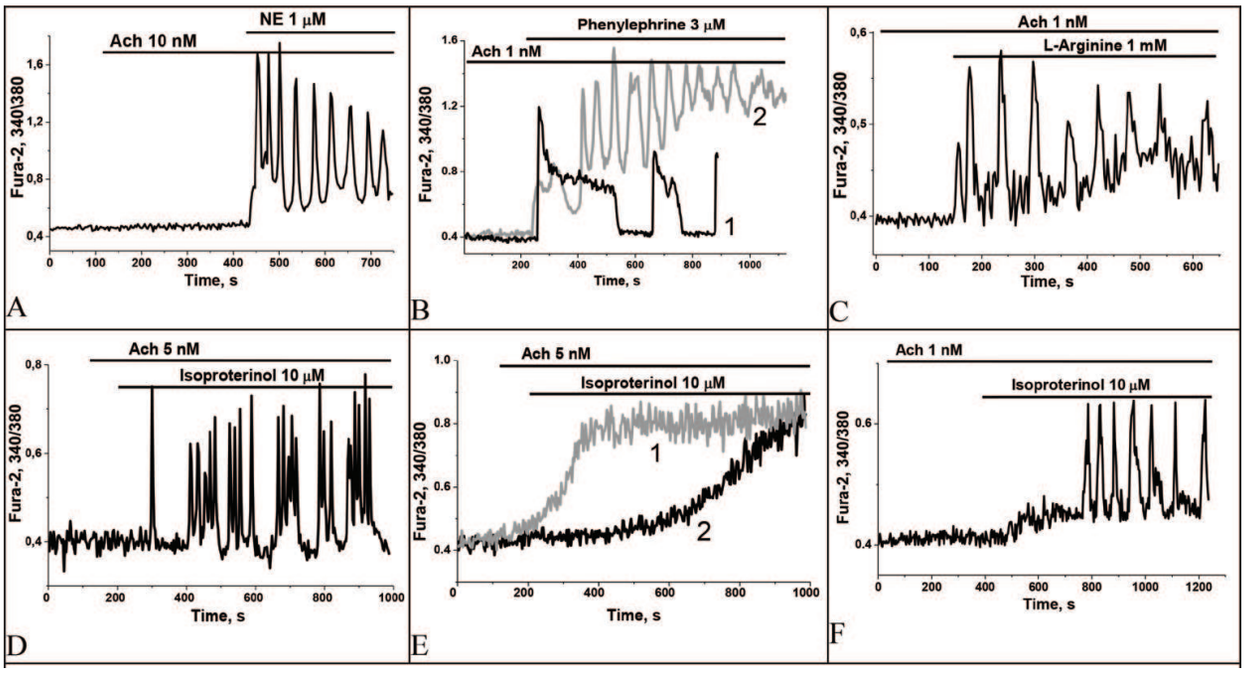

Figure 4. Signaling interplay and sensitivity. Synergistic action of low concentrations of ACh and NE. A–C: Interplay of Gβγ –subunits of G- proteins of GPCR. Ca^{2+}- oscillations and triggering regime (4B, record 2) produced by combined action of low concentrations of ACh and NE, ACh and phenylephrine, ACh and L-arginine, correspondingly. Record 1 at B represents an example of relaxation oscillations. D–F: Interplay of Gβγ- subunits of Gq- proteins (ACh) and of *β1–3 - AR* (isoproterenol). Complex oscillations (D), triggering regimes (E) and Hopf bifurcation (F) produced by combined action of low concentrations of ACh and isoproterenol. Description in the text. Results from: Turovsky et al., submitted to publisher.

3. Loss of rhythmic activity and suppression of mRNA expression for the proteins of Ca^{2+}- signaling systems at obesity

3.1. Influence of cell size on rhythmic activity of adipocytes of healthy animals

Preadipocytes isolated from healthy male mice, growing on high-glucose DMEM medium, become differentiated to the ninth day of culture (9DIV). Mature adipocytes represent heterogeneous populations of cells, which is characterized by different cell size, in dependence of number of lipid droplets inclusion [57]. We evaluated cell size by measuring the area (S) occupied by the cell. Small adipocytes, having S ≈ 300–400 μm^2, generated fast regular monomodal Ca^{2+}- oscillations with periods from 5 to 75 s in response to ACh or NE [57]. Such cells accounted for 10–15% of all monitored cells in culture and had few small lipid droplets. More than 50% of the cells had cellular size S ≈ 500–900 μm^2. Rest of cells (15–20%) with S ≤ 1100 μm^2 had several big lipid droplets or one lipid inclusion, which might occupy from 70 to 90% of the cell volume. ACh and NE elicited complex multimodal Ca^{2+} oscillations with periods from 100 to 300 s in the cells with S ≥ 600 μm^2. Similar results, characterizing correlation of cell size with the shape and period of oscillations, have been registered for insulin [59]. Results presented in lower part of **Table 1**(fifth row) indicate that, independently of agonist used, cultured cells may generate Ca^{2+} oscillations in the ranges of periods from 5–60 s to 400–500 s.

Rhythmicity disappeared in hypertrophied adipocytes with S ≥ 1200 μm², which may respond to the application of high doses of ACh or NE (20–30 μM) only by Ca^{2+} spikes or slow Ca^{2+} accumulation [57]. These observations may indicate that uncontrolled hypertrophy and corresponding cytoplasm shortage might predispose to loss of rhythmic processes in adipocytes and to the development of general hormonal resistance in WAT cells.

3.2. Impact of obesity on Ca^{2+} signaling systems of adipocytes

3.2.1. Model of obesity

We used 6 to 8 month course of high-fat feeding, based on the addition of pork lard (200–300 mg/day/animal) to standard chow of rodents, taking in experiments 7–8 month old mice. This model is described briefly in Appendix. Obese 6–7-month-old mice had elevated levels of glucose in blood in a fasted state (7–9 mM), raised arterial pressure (130–150 mm Hg), and macromolecular liver steatosis (Grishina et al., submitted to publisher).

3.2.2. Ca^{2+} signaling in hypertrophied primary adipocytes and cultured cells

Isolated primary epididymal adipocytes of medium size (S ≈ 6000–7500 μm²) had approximately 1–5% of cytoplasm (**Figure 5B** and **C**), which looks like bright oreol around of adipocyte. Most of these cells, being attached to cover glass by Cell-Tak adhesive, cannot generate Ca^{2+} signal in response to added high concentrations of ACh (**Figure 5D**). However, most of hypertrophied cells, having spots of cytoplasm, still preserve the ability to respond to added Ca^{2+} (**Figure 5E** and **F**) or ionomycin (**Figure 5D**). This kind of nonresponsiveness to ACh might characterize general hormonal resistance of hypertrophied eWAT cells in obese state.

Preadipocytes isolated from fat pads of obese animals cannot grow on glucose, being adapted to use long-chain fatty acids (LCFA) incoming from the blood. Incubation of preadipocytes with 100 nM of L-palmitoylcarnitine (PC) and standard high-glucose DMEM provides required conditions for cell differentiation and lipid accumulation. ACh, NE, and insulin may evoke weak Ca^{2+} signals in some part of cultured cells. Oscillations and standard amplitude Ca^{2+} spikes have never been observed in this kind of cell (Turovsky et al., submitted to publisher). These radical alterations in Ca^{2+}-signaling and hormonal sensitivity, registered in cultured cells of obese animals, may depend on radical alterations of mRNA expression for the enzymes and channels of both Ca^{2+} signaling systems (PLC-RG and NOS-RG).

3.2.3. Impact of LCFA on mRNA expression of cultured cells

Our preliminary results, obtained with the application of real-time PCR analysis (see Appendix), revealed significant depression of mRNA expression in cultured adipocytes of obese animals in comparison with the cells, which have been isolated from healthy animals. Results presented in **Figure 6** (gray columns) demonstrate that cultures grown on the medium containing 100 nM of PC have 3–5 fold lowering of the expression of: Ca^{2+}-dependent genes (of NFAT, NF*k*B); genes of proteins involved in energy and lipid metabolism (citrate synthase (CS) and HSL); marker genes of SIRT1, AMPK, PI3Kγ, and of eukaryotic translation initiation factor 2 alpha kinase 3 (PERK). As for NOS-RG and PLC-RG, the expression of mRNA of eNOS (NOS3), CD38, and RyR3 had fallen 10 times or more. The gene of IP3R isoform 3 (IP3R3) was

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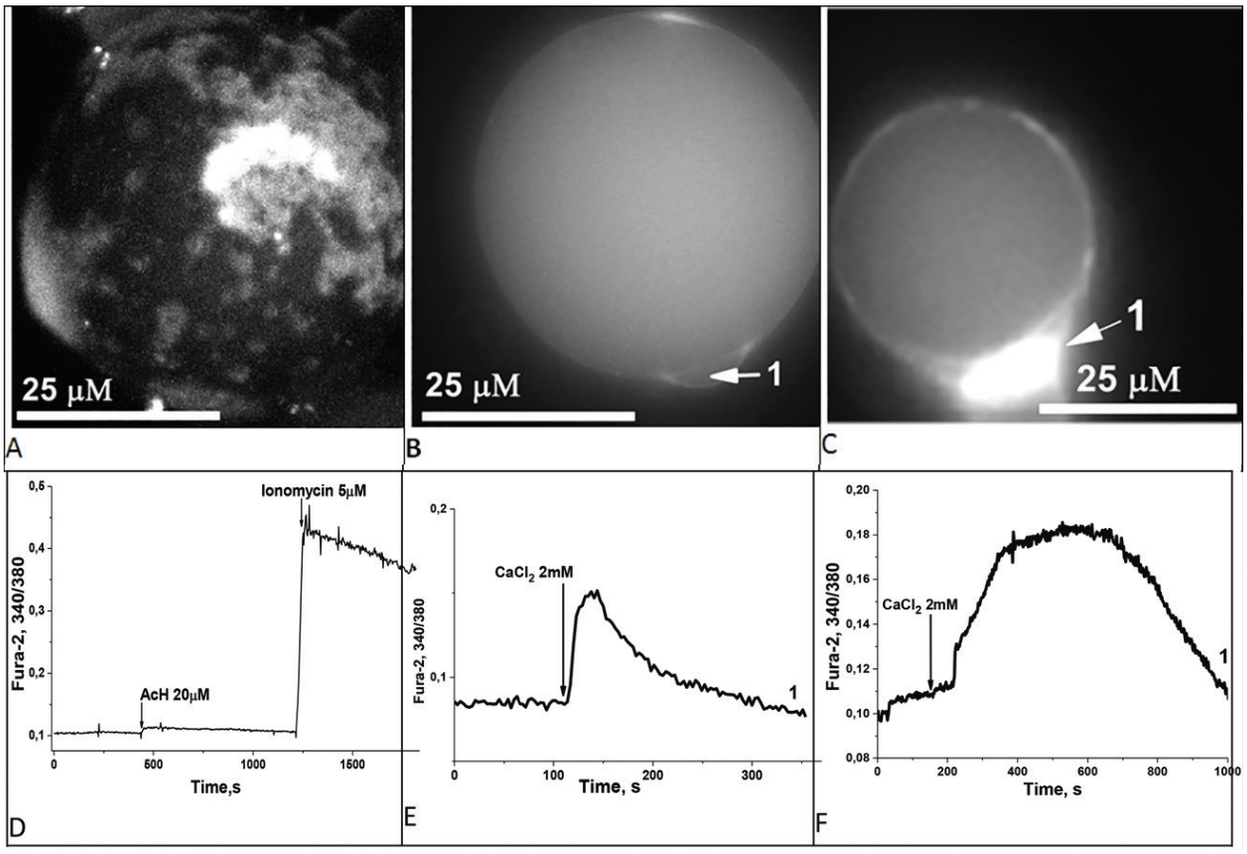

Figure 5. The shape and Ca^{2+} responses of primary hypertrophied adipocytes of obese mice. (A) confocal image of 3D reconstruction of primary hypertrophied adipocyte of obese mice (front view of one cell). The projection describes general uneven distribution of the cytoplasm in the cell loaded by Fluo-4. Fluorescent space corresponds to the part of cell occupied by the cytoplasm (up to 1–3% of total cell volume), while the bulk of the cell is engaged by fat droplet. (B, C) bright fluorescent areas around hypertrophied cells visualize the cytoplasm by fura-2 dye and are numbered as 1. Intracellular Ca^{2+} responses (Fura-2, ratio) from these areas, which were elicit by external Ca^{2+}, are presented at panels E and F, correspondingly. Panel D describes rare type of Ca^{2+} response evoked by ACh. Panel D also demonstrates ACh resistance of hypertrophied cell. Though, the response to ionomycin is preserved. From Sergeev et al., submitted for publisher.

most resistant to toxic action of very low dose of PC. The expression of inducible NOS (NOS2) mRNA was measured at the level of detection (Grishina et al., submitted to publisher).

Incubation of preadipocytes (isolated from obese animals) with 1 μM of PC revealed dramatic suppression of mRNA expression for all genes analyzed in cultured cells (9DIV). The expression of marker genes of NOS3 and RyR3 was not observed (marked in **Figure 6** as *). These results support widely distributed viewpoint on LCFA toxicity and indicate an important role of LCFA in the development of "adiposopathy". Observed radical alterations in mRNA expression, especially for the proteins involved in the functioning of NOS-RG and PLC-RG, may mean that earlier discussed mechanisms of autoregulation and cross-control of both Ca^{2+} signaling systems are being lost under chronic toxic action of low concentrations of LCFA. This state of both systems may be characterized as absolutely deregulated state.

3.2.4. Expression of mRNA in eWAT of obese animals

In comparison with fat pads of age-matched healthy animals, eWAT of obese male mice is characterized by considerable down-regulation of the expression for all genes examined

(**Figure 7**). These alterations have some qualitative similarities with the results observed in cultured cells, especially with respect to the changes of expression of marker genes for the proteins of NOS-RG. Fat pads of obese animals have more than 8–12 times lowered expression of PKG1, PKG2, and eNOS genes. The expression of genes for RyR2 and RyR3 was under the level of detection. Observed significant down-regulation of IP3R1 and IP3R2 genes may mean that both Ca^{2+} signaling systems are in deregulated states. Considering NOS-RG as the system, which integrates hormonal signals involved in the control of NO bioavailability, we may conclude that the application of insulin, NE, ANP, and so on might be ineffective to rise NO level and PKG1 activity in "sick" fat depots of obese animals.

3.2.5. Benefit and disadvantages of physical activity in the treatment of obesity

Taking into account the benefit of physical activity in the treatment of obesity, we have applied a very low-intensity treadmill running program (6 weeks, 10 min/day) to treat diabetic overweight mice (56.2+/− 5.7 g. wt) in combination with animals treatment with NaCl (control group) or complex preparation, addressed for the treatment of liver diseases and hepatic encephalopathy [88]. However, in a control group, 8 of 20 diabetic mice treated with NaCl have died within first week of adaptation period due to exercise intolerance. Survivors were characterized by marked improvement in blood glucose and lipid profiles and in liver mRNA expression of all genes examined (of PLC-RG and NOS-RG). In comparison with control group, all animals treated with complex preparation tolerated exercise program well and showed further improvement in blood lipid profiles and mRNA expression [89]. Taking all this into account, we might speculate that application of various exercise programs to treat obese patients should be combined with the use of some performance-enhancing drugs, or drugs addressed to support liver and cardiovascular system, and so on.

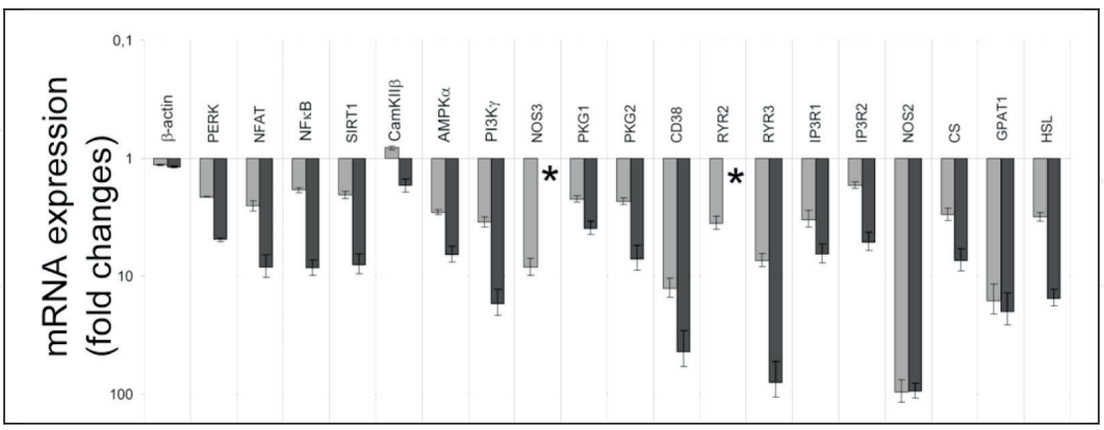

Figure 6. Down regulation of marker genes expression in cultured adipocytes of obese male mice grown in presence of L-palmitoylcarnitine. On the plot are resented: Ca^{2+}-dependent genes NFAT and NF*k*B, genes of PERK and proteins of NOS-RG, IP3R (IP3R1,2 - subtypes 1, 2), CamKIIβ, AMPKα, and of energy and lipid metabolism (citrate synthase (CS), GPAT1, HSL), β- actin and of inducible NOS (NOS2). Gene of GAPDH is used as reference gene. Mean expression in control adipocytes from healthy animals was set as 100%. Error bars indicate SD. Gray and black columns correspond to cultured adipocytes grown in presence of 100 nM and 1 μM of L-palmitoylcarnitine, correspondingly.* indicate the absence of mRNA expression for eNOS (NOS3) and of subtype 3 of RyR (RyR3). Horizontal line marked at the level 1 indicates baseline gene expression. N = 4, number of cultures in each group.

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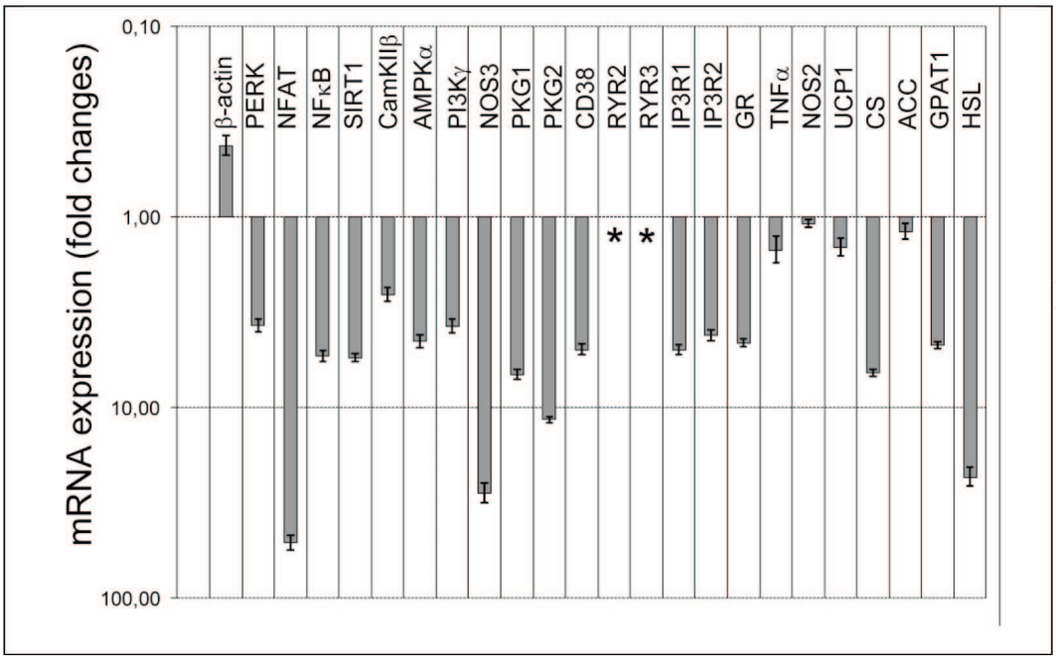

Figure 7. Down regulation of marker genes expression in eWAT of obese male mice. Presented Ca^{2+}-dependent genes NFAT and NF*k*B, genes of PERK and proteins of NOS-RG, IP3R (IP3R- subtypes 1, 2), glutathione reductase (GR), uncoupling protein 1 (UCP1), tumor necrosis factor α (TNFα), acetyl coenzyme a carboxylase (ACC), CamKIIβ, AMPK, and of energy and lipid metabolism (citrate synthase (CS), GPAT1, HSL), β-actin and NOS2. Gene of GAPDH is used as reference gene. Mean expression in control adipocytes from healthy 8 month old animals was set as 100%. Error bars indicate SD. N = 5, number of animals in each group.* indicate the absence of mRNA expression for both subtypes of RyR (RyR2, 3).

4. Conclusions

The main aim of our chapter was to reconstruct core elements of the Ca^{2+} signaling system of adipocytes and to demonstrate that this complex multivariable system cannot be divided on separate parts, independently controlled by various hormones and/or neurotransmitters. Having multiple feedbacks and cross-controls (**Figures 1** and **2**), this system makes interdependent the concentrations of all second messengers and the activities of various kinases. For example, considering lipogenic and antilipolytic action of insulin [7, 8], we have to take into account its lipolytic action. Insulin increases NO bioavailability and PKG1 activity by activating NOS-RG. The same effect may be produced by CCK, BK, Ang II (see **Table 1**). Reliability of NOS-RG, receptors' signaling interplay, and amplification of signals create important properties of Ca^{2+} signaling system, providing integration of hormonal signals at their low concentrations (**Figure 4**). This is especially important with respect to ACh.

Parasympathetic control of WAT is still under the question [90]. However, in our experiments ACh evokes marked Ca^{2+} and NO responses in cultured cells, implicating NOS-RG [61]. Some sensitivity to ACh is preserved in primary hypertrophied adipocytes ([57] and **Figure 5D**). Due to that, possible role of ACh in the control of WAT metabolism requires further investigations.

Gradual loss of rhythmic activity and the appearance of hormonal resistance, which are observed in hypertrophied cultured cells and in primary adipocytes isolated from obese animals, may be considered as markers of cell viability in the progress of pathologic process. Similar alterations in rhythmicity and resistance to ACh and NE have been registered in aorta rings isolated from obese and diabetic rats [87, 89].

Loss of rhythmicity in adipocytes is based on the alterations in enzyme activities and loss of feedback control of PLC-RG and NOS-RG, due to marked alterations in mRNA expression of corresponding genes (**Figures 6** and **7**). Our preliminary results indicate that qualitatively similar alterations in gene expression are observed in the liver of obese and diabetic mice [90].

All this may indicate universal mechanisms resulting in deregulation of metabolic and signaling systems in various organs and tissues.

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Conflict of interest

Authors declare no conflict of interests.

A. Appendix

Gene expression analysis

Gene expression in cultured adipocytes and in eWAT was performed using real-time PCR (Applied Biosystems 7300) with TagMan Universal Master Mix II, no UNG (Applied Biosystems). Total RNA was isolated with TRIzol (Invitrogen). RNA was quantified by Qubit® RNA BR Assay Kit (Molecular Probes, Eugene, OR) and cDNA was synthesized from 5 μg of total RNA using a reverse transcription system with random primers (Sileks, Russia). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH was used as reference gene. All results were normalized to GAPDH mRNA expression. Fold difference in each gene expression was calculated as $2^{-\Delta\Delta Ct}$. ΔΔCt were calculated relative to corresponding gene of control adipocytes grown on glucose, or of control healthy age-matched white male mice.

Animal model of obesity and type 2 diabetes

Animal model of obesity and type 2 diabetes (T2D), described for rats previously [87], was used in present experiments. This model is heterogeneous, like those presented by Duval et al. [5]. We used 6–8 month course of high-fat feeding, based on the addition of pork lard

(200–300 mg/day/animal) to standard chow of rodents, taking in experiments 7–8-week-old mice. Obese 6–7-month-old fat-responsive mice had elevated level of glucose in blood in fasten state (7–9 mM), raised arterial pressure (AP = 130–150 mm Hg) and macromolecular liver steatosis (Grishina et al., submitted for publisher). The animals with advanced T2D (9–10 month) have been characterized by: AP = 140–170 mm Hg, fasting glucose level of 12.1 ± 1.8 mM (SD), insulin of 2.9 ± 1.3 ng/ml (SD) and venous blood ammonia higher than 100–140 μM, liver fibrosis or even cirrhosis. Dysfunctional preadipocytes, isolated from "sick" epididymal fat depots of diabetic mice, were characterized by inability to proliferate (Turovsky et al., submitted for publisher).

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