REVIEWS

Ultradian Rhythms in Cell Population. Problem of Synchronization

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The studies demonstrating cooperativity of cells in synchronization of their activity are reviewed. The total ultradian rhythm in a cell culture is taken as a marker of synchronization. Self-synchronization of interacting oscillators has been demonstrated in experiments. Special attention is paid to the mechanisms underlying formation of ultradian rhythms of protein synthesis in a culture of rat hepatocytes. Formation of populational rhythm in these cells is a function of cell density and time of culturing without replacing growth medium (conditioning). The addition of some individual exogenous gangliosides to the culture medium simulates the effects of conditioning. Immunocytochemical studies showed that intracellular expression of ganglioside determinants is enhanced during the conditioning of culture medium. Exchange of gangliosides between cells and their intracellular accumulation may be the first step in synchronization of cell activity. Synchronization of the protein synthesis oscillations was demonstrated in hepatocytes *in situ* in a denervated liver. From these observations and literature data it is concluded that self-synchronization of cellular activity is a fundamental regulatory mechanism of organ functioning which operates in line with regulatory systems acting at the organism level.

Key Words: *ultradian rhythms; synchronization; protein synthesis; gangliosides; liver; hepatocytes*

The review is based on the report delivered March 12, 1997 at the Conference organized by the Institute of Developmental Biology (Russian Academy of Sciences) and Russian Medical University to commemorate the 100th anniversary of Academician G. K. Khrushchov and concerns recent studies carried out at the laboratory founded by G. K. Khrushchov. Almost four decades ago we discovered oscillations of an about one-hour period in the kinetics of cellular protein mass. Then ultradian rhythms were identified by others in about 20 cell functions and properties, such as protein synthesis, enzyme activity, intracellular ATP and cAMP contents, protein secretion, cellular respiration, and sometimes cell size and mass [26,27]. Various ultradian rhythms were revealed in differentiated cells, for example, nervous and glandular, and in blastomeres. These rhythms exist in the cells of mammals, echinoderms, crustaceouses, mollusks, unicellate organisms, and bacteria. In mammalian cells, ultradian rhythms have been observed both *in situ* in cell cultures (both primary and secondary). Most recent studies were performed on cell cultures. Investigation of any rhythm in a cell culture raises a question: why the rhythms has been revealed? The answer to this question has been found in the course of the study of intercellular interactions in a hepatocyte culture.

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Total Rhythms of Cellular Population is a Result of Self-Synchronization of Oscillations. An individual cell can be an oscillator. Ultradian oscillations occur in ultraviolet absorbance (the protein absorbance range) in crayfish mechanoreceptor neurons [14], cytoplasmic pH of individual cell in persisting culture [16], and energy potential of hepatocytes as revealed by *in situ* staining with rhodamine 123 [15]. It seems quite reasonable to suggest that populational rhythm ("leveling") is impossible in cell culture, i.e., in the absence of central (nervous and/or hormonal) synchronizing signals, implying that cells generate oscillations of different phases which quench each other. However, the rhythm was recorded. What synchronizes the oscillations of individual cells?

We put forward a hypothesis on self-synchronization in cell population about 20 years ago [26]. This hypothesis was based on mathematical models of interacting oscillators [12,39]. There was evidence on synchronization of oscillations in biological objects. For instance, contractions of isolated cardiomyocytes varying in frequency were replaced by a 1-sec rhythm after cell-to-cell contacts had been established [11]. Synchronization of size oscillations was observed in contacting glial cells [19]. Glycolysis oscillations with 1-min periodicity in different yeast cultures were replaced by a unique rhythm after mixing these cultures, but were not quenched as a result of simple summation of the oscillations [23,29,40].

While studying ultradian rhythms of the intensity of protein synthesis in cultured rat hepatocytes, we observed self-synchronization of oscillations and identified some mechanisms of intercellular cooperation.

We studied the mechanisms responsible for self-synchronization of intensity of protein synthesis (by incorporation of radiolabeled leucine taking into account the intracellular leucine pool) in cell cultured with phase-opposite rhythms of protein synthesis [8]. Suspension of rat hepatocytes (cells were isolated from one liver) was divided into two portions. One portion was cooled for 20 min and then warmed to normal temperature. This shifted the rhythm by about a phase. Other portion was placed in a thermostat at 37°C. Comparison of kinetics of protein synthesis in cultures grown from these suspensions revealed that the maxima of protein synthesis often coincide. Arithmetic summation of kinetic curves yielded a straight line, pointing to antiphase oscillations. This line reflects the situation when intercellular interactions are absent: summation and quenching of oscillations in the intensity of protein synthesis. However, when protein synthesis intensities in cooled and intact cultures are compared, rhythm has been always observed. Thus, self-synchronization always occurs in a cell population. The rhythm can serve as a marker of synchronization of cell population activity.

Synchronization of Oscillations of the Protein Synthesis Intensity Occurs in Conditioned Medium. The rhythm has been observed in a cell monolayer, contacting cells, and cells with weak contacts, but not in diluted cultures growing in fresh medium and in preconfluent cultures [9]. The rhythm of protein synthesis was observed after individual hepatocytes had been incubated with confluent culture for 30 min [7] or after replacement of culture medium by culture medium conditioned by hepatocyte monolayer. This observation suggests that synchronizing factor is secreted in the culture medium.

In situ or in confluent culture of contacting cells cellular functions are determined by microenvironment. In this case a similar state of the cells is due to a common molecular and ionic intercellular space. Asynchronous oscillations of some cells are rapidly quenched owing to activity of neighboring cells that determine the properties of a common growth medium. It can be suggested that in cultures with weak cell-to-cell contacts signaling molecules detach from the plasma membrane of one cell and after specific interaction with the plasma membrane of other cell coordinate its function. Presumably, the concentration of synchronizing factor in diluted cultures is insufficient to provide effective intercellular interactions. Coculturing increases the concentration of signaling molecules in the close vicinity of individual cells, thus synchronizing their activity. Such a synchronization is impossible in fresh medium. What compound can act as a synchronizing factor or at least initiate synchronization? From analysis of the literature data we have hypothesized that gangliosides can fulfil the synchronizing function. This hypothesis was confirmed experimentally.

Addition of Gangliosides to Fresh Culture Medium Synchronizes Oscillations of the Protein Synthesis Intensity in Individual Cells. Synchronization of oscillations of protein synthesis intensity in dilutes cultures was observed after the addition of gangliosides to the culture medium [5].

Gangliosides are present in all mammalian cells and perform important physiological functions [13, 22,24,25,28,30,33-36,41,43]. They are concentrated primarily in the plasma membrane and are also present in the cytoplasm. Gangliosides can detach with a fragment of plasma membrane from one cell and be incorporated in the plasma membrane of another. Gangliosides act as receptors, binding with some toxins, or as coreceptors, modulating the sensitivity of receptors for neurotransmitters, insulin, fibronectin, and growth factors. Gangliosides and products of their degradation modulate the activities of

protein kinase and adenylate cyclase. They modify calcium inflow and outflow and its mobilization from intracellular stores.

We observed synchronization of protein synthesis in diluted cultures of rat hepatocytes after the addition of total ganglioside fraction isolated from bovine brain [5]. The fraction contained 20% GM1, 40% GD1a, 16% GD1b, 20% CT1b, and about 4% minor higher gangliosides.

Thus, gangliosides initiate the processes synchronizing oscillations in cell culture.

Individual Gangliosides Provide Synchronization. It was then demonstrated that individual gangliosides synchronize the protein synthesis oscillations [6]. The rhythm was observed after the addition of GM1 to a fresh growth medium of diluted culture to a final concentration of 0.1-0.2 μM. Synchronizing effect was also produced by micromolar concentrations of GD1a, but not by GD1b, GT1b, and GM3. GM3 is represented by a minor fraction in bovine brain; however, in rat liver it amounts for more than 30%.

A question arises: whether the concentration of gangliosides increases in cells growing in conditioned medium, i.e., in cells with synchronous rhythm of protein synthesis?

Gangliosides Are Accumulated in Cells upon Conditioning of Culture Medium. Expression of ganglioside determinants was assessed by indirect immunocytochemical methods using affinity-purified anti-GM1 and anti-GM3 rabbit polyclonal antibodies [6]. A considerable increase in the intensity of immunofluorescence was observed in confluent cultures of hepatocytes and in nonconfluent cultures in conditioned growth medium. Individual cells became fluorescent after a 30-min incubation in the presence of total gangliosides or GM1. This coincided with synchronized protein synthesis. Ganglioside expression was weak in thoroughly washed 24-h monolayer and preconfluent cultures. Similar changes in the culture medium conditioning, intracellular ganglioside accumulation, and rhythmic activity of cell population confirm the hypothesis that gangliosides fulfil the synchronizing function.

Immunocytochemical studies corroborate the concept on critical concentration of synchronizing factors providing cooperative cellular activity. Accumulation of gangliosides in the culture medium proved to be a function of both cell density and duration of culturing. Long-term (>24 h) culturing at a low cell density (diluted suspensions) without replacing growth medium (conditioning) stimulated the expression of gangliosides by noncontacting cells. In these cultures, the protein synthesis rhythm was observed in conditioned but not in fresh medium. Fluorescent cells were present in cultures with both conditioned and fresh medium, indicating that gangliosides are rapidly accumulated in close vicinity of cultured cells and then concentrated on their plasma membranes.

It is likely that gangliosides only initiate synchronization. What synchronizes the cells?

What Are Potential Intracellular Factors of Synchronization? So far this question remains without answer. It should be remembered that even a weak influence spreading over a considerable portion of cell population can synchronize cellular functions, including protein synthesis. Similar to a short-term cooling, which inhibited protein synthesis and shifted the phase of oscillations in most cells, other influences spreading over many cells may synchronize protein synthesis. Proceeding from the properties of gangliosides, such a "leveling" influence modifies ion fluxes, is transient, and spreads over a considerable number of cells in the population. For instance, repeated intense transient mobilization of calcium from cytoplasmic depots is induced by some neurotransmitters and hormones [1]. The process spreads over majority of cells or even all cells and, consequently, synchronizing stimulus may appear.

Gangliosides change intracellular concentration of free calcium by modifying calcium transport or by binding with calmodulin [37]. The contribution of ATP and cAMP to synchronization of cell population should be evaluated in further investigations.

The effect of gangliosides and their metabolites can be mediated by protein kinases. These enzymes synchronize the activity of cell population by reversible phosphorylation of cellular proteins on the plasma membrane and in the cell [28,31,32]. They stimulate phosphorylation of some proteins and inhibits phosphorylation of others. However, the spread of influence but not its direction is important for synchronization.

Intercellular Interactions Leading to Synchronization of Protein Synthesis Occur Both *In Situ* and in Cell Culture. In order to find out whether cellular activity is synchronized *in situ*, we studied the kinetics of protein synthesis in denervated liver. We attempted to answer the question whether the rhythm of protein synthesis is preserved in the liver after elimination of nervous regulation.

Synchronization of protein synthesis was observed in experiments with the maximum possible denervation of rat liver (bilateral cut of the vagus and desympathization), when nervous signals were blocked, liver circulation was changed, and hormonal influences were altered [4]. The rhythm profile was the same as in intact liver and hepatocyte cultures.

Synchronization of Protein Synthesis Occurs in Different Cells of Anatomical Unit. Inphase oscil-

lations of protein synthesis were originally revealed by autoradiography in different layers of mouse retina (synchronization with light stimuli) [3]. Synchronization of cellular rhythms was also observed in bioptates of human gastric mucosa [10,17,20]. Gastric mucosa consists of epithelium (superficial and glandular), connective tissue layer with various cell types, and muscular layer. Therefore, it is normal to expect that protein synthesis in it is not synchronized. In fact, no rhythm was observed in relatively normal mucosa during long remission of peptic ulcer.

However, in acute stage of duodenal ulcer protein synthesis in gastric mucosa is enhanced and synchronized. Presumably, pathological processes in the duodenum modify the state of gastric mucosa so that protein synthesis is enhanced and its phases are synchronized in cells that do not contact with each other but are subjected to similar exogenous influences. This leads to the development of a common rhythm.

Thus, self-synchronization of protein synthesis was demonstrated in cultured hepatocytes. Since the rhythm of protein synthesis has been observed in other cell cultures together with numerous other ultradian rhythms, the principle of self-synchronization is common for cell interactions. It was demonstrated that intercellular interactions upon synchronization of protein synthesis occur via the culture medium. Synchronization occurs in conditioned medium, because cells release synchronizing factors. Micromolar concentrations of exogenous gangliosides reproduce the effects of synchronization. Since gangliosides are constantly detached from the plasma membrane, saturation of the culture medium or extracellular tissue *in situ* with gangliosides is an important, although not unique, biochemical factor of conditioning (formation of a common medium for the entire cell population). Presumably, gangliosides trigger the cascade leading to synchronization of activity of a cell population. Modulations of ion concentrations and protein phosphorylation with activation or inhibition of key enzymes are tentative intracellular mechanisms responsible for synchronization.

Experiments on denervated liver showed that cellular communication are preserved *in situ*, suggesting that synchronization of cell activity is a result of direct intercellular interactions.

So far, the rhythm of protein synthesis was regarded only as a marker of synchronization of cell activity. However, the significance of ultradian rhythms is comparable to that of diurnal rhythms [42]. Ultradian rhythms have been identified in a wide range of biological objects; they contribute to numerous physiological functions and to diurnal rhythms of these functions and are a component of the total time control in the organism. It was hypothesized that ultradian rhythms have a fractal nature [42]. The similarity between ultradian rhythms and fractals has been recently substantiated [2]. These rhythms are highly irregular, determinant, and resistant to exogenous stimuli (including temperature). They preserve their structure upon changes in the time scale. These properties point to a high reliability of biological systems [38]. Multiplicity and irregularity are important components of temporal tissue organization and as a condition of their informational structure [18]. In morphology and histophysiology, the principle of multiplicity and polymerism of microanatomic structure has been regarded as determinants of the regulation of functions and physiological regeneration [21].

Ultradian rhythms are also important from the viewpoint of their involvement into the functioning of internal organs and entire organism. Integral ultradian rhythms were observed in respiration, heart rate, dynamics of numerous hormones, cerebral activity, motor activity, and behavior of animals and humans [42]. Since the sings of "fractal behavior" have been observed for the rhythms of some organs, it can be suggested that in addition to nervous and hormonal regulation of rhythms and the corresponding functions of various organs, intrinsic cellular mechanism of synchronization are preserved in mammals.

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