========= **REVIEWS** =========

# Evolution of Ca²⁺-Signaling Mechanisms. Role of Calcium Ions in Signal Transduction in Lower Eukaryotes

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**Abstract**—This review summarizes current concepts of $Ca^{2+}$-signaling mechanisms in unicellular eukaryotes. Pathways of activation and transduction of $Ca^{2+}$-signal are analyzed and the role of $Ca^{2+}$ in regulation of cellular physiological processes is considered. A special attention is paid to evolutionary aspects of participation of $Ca^{2+}$ ions and $Ca^{2+}$-receptor proteins in regulation of intracellular processes.

## INTRODUCTION

Mechanisms of intracellular signaling involving calcium ions as a second messenger have an ancient evolutionary origin. Even bacterial cells have been shown to contain such important functional components of the information intracellular systems as $Ca^{2+}$-channels, primary and secondary transmembrane $Ca^{2+}$-carriers, calmodulin (CaM)-like proteins mediating participation of $Ca^{2+}$ in regulation of cell division, growth, and chemotaxis [1].

The tendency for use of $Ca^{2+}$ in processes of intracellular signaling was further developed in cells of the lower eukaryotes (protists). In protists, a multicomponent system for accumulation, regulation, and utilization of intracellular $Ca^{2+}$ sources is formed for generation of the calcium signal. This system is similar to that of higher eukaryotes, it includes mechanisms of electro- and receptor-controlled $Ca^{2+}$ input from extracellular fluid, ways of $Ca^{2+}$ mobilization from intracellular stores with the aid of inositol-1,4,5-triphosphate ($InsP_3$), arachidonic acid, and cyclic ADP-ribose as well as the mechanism of the "$Ca^{2+}$-induced $Ca^{2+}$-release" (CICR) type.

In protist cells, like in cells of the higher organized animals, the mechanisms of intracellular signaling of $Ca^{2+}$ as a second messenger consist of structural and functional elements capable for reception, modulation, and translation of the triggering calcium signal. As the cytosol $Ca^{2+}$ acceptors, not only receptor proteins of the calmodulin type serve, but also signal proteins that are a part of the signal protein kinase cascades.

Comparative analysis of the complex $Ca^{2+}$-signaling mechanisms in cells of the lower and higher eukaryotes revealed many similar features indicating the universal and single principles underlying the structural-functional organization of all eukaryote $Ca^{2+}$-dependent signaling systems, although there also are differences, whose nature appears to be due to peculiarities of molecular evolution of the signal mechanisms.

By the present time, some molecular $Ca^{2+}$-signaling mechanisms in the lower eukaryotes have already been deciphered. Pathways of activation and propagation of the $Ca^{2+}$-signal are described, proteins modulating activity of calcium signal are determined, and the role of $Ca^{2+}$ in regulation of physiological processes in protist cells is established. However, in the evolutionary aspect, the

problem of $Ca^{2+}$-signaling has not been earlier considered.

This review summarizes data on evolution of the $Ca^{2+}$-signaling mechanisms that have become the ground for development of the $Ca^{2+}$-messenger system in cells of Metazoa.

## MECHANISMS OF FORMATION OF $CA^{2+}$-SIGNAL IN THE LOWER EUKARYOTE CELLS

An increase of the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) is a trigger starting signal for the beginning of numerous physiological processes in the cells both of the higher animals and of microorganisms including prokaryotes [1].

The triggering $Ca^{2+}$ signal is formed on the surface of the plasma membrane (PM) during a sharp change of environmental conditions as well as under the effect of mechanic, stress, and alimentary factors and is propagated into a cell through specialized structures—receptors and/or ion channels. A considerable amount of information coming into cells of the lower eukaryotes is realized via $Ca^{2+}$ channels that are included in a specialized regulatory-transport system providing reception, transduction, and transmission of the $Ca^{2+}$ signal. Some of them will be considered below.

*$Ca^{2+}$-channels.* The presence of $Ca^{2+}$ channels has been established in cells of many species of unicellular eukaryotes, fungi, and algae. In these microorganisms, evidence for the existence of several types of $Ca^{2+}$ channels has been experimentally confirmed; they differ by pharmacological characteristics and by location from the $Ca^{2+}$ channels in prokaryote and Metazoa cells.

In cells of eukaryotes, $Ca^{2+}$ channels are a part of the transduction systems using not only external, but also internal $Ca^{2+}$ sources for formation of the calcium signal that represents a local rise in $[Ca^{2+}]_i$). For realization of fast cellular reactions, extracellular $Ca^{2+}$ is preferentially used. This mechanism of the potential-controlled $Ca^{2+}$ input operates, when cells respond to depolarization of the plasma membrane (PM) by an increase in the $Ca^{2+}$ input through the potential-controlled $Ca^{2+}$ channels (Ca(v)-channels). This mechanism is characteristic of electrically excitable cells of multicellular organisms [2], some protists (infuso-

ria) [3, 4], and the lower eukaryotes [3, 5]. To provide initiation of slow metabolic and signal processes, intracellular $Ca^{2+}$ sources as a rule are used. In this case, the external triggering signals realize their action through activation of the receptor-controlled $Ca^{2+}$ channels located in membranes of subcellular organelles, such as Golgi complex, endoplasmic reticulum, and vacuoles. This mechanism of the receptor-dependent $Ca^{2+}$ mobilization is typical of unexcitable Metazoa cells and most cells of the lower eukaryotes.

*Potential-gated calcium channels.* The Ca(v)-channels are found in all types of electro-excitable cells of multicellular organisms and are characterized by that they are activated during PM depolarization, whereas at the rest potential (approximately from $-70$ to $-80$ mV) they are in the inactive state. In mammalian cells, four variants of Ca(v)-channels, L-, T-, N- and P-types, have been described in the greatest detail; they differ in location, potential-dependence, conductivity, sensitivity to pharmacological agents, and some other characteristics [2, 6, 7]. In cells of homoiothermal animals, the most numerous are Ca(v)-channels of the L-type that on activation, preserve this state relatively long [7, 8].

The Ca(v)-channels have also been revealed in cells of the lower eukaryotes. There are data for the existence of electro-gated channels in the PM of ciliated infusoria Paramecium [2, 4, 9], in tonoplasts of the yeast *Saccharomyces cerevisiae* [5, 10], in the PM of *Euglena gracilis* and *Astasia longa* [11]. The electro-gated channels have been studied in the greatest detail in Paramecium [2, 12, 13].

Jennings [14] observed behavior of the infusoria *Paramecium caudatum* responding to contact, light, elevation of temperature, and chemical stimulation. These observations stimulated subsequent neurobiological investigations that have enabled concluding that infusorian cells are unique forms combining simultaneously functions both of biosensor and of effector; therefore, they were figuratively called "swimming sensor cells" or "swimming neurons" [12]. The Paramecium body is covered with thousands of cilia containing receptors and ion channels including Ca(v)-channels. Like classical Ca(v)-channels of vertebrates, the electro-controlling calcium channels in infusoria are activated by some cations, organic repellents, me-

chanical stimuli, elevated environmental temperature, i.e., factors initiating PM depolarization [15]. The membrane depolarization, in turn, evokes the action potential opening Ca(v) channels for Ca$^{2+}$ input. The [Ca$^{2+}$]$_i$ increases and the swimming cell behavior changes practically instantaneously, which is manifested as the physiological "reaction of escape" caused by reversion of ciliary beating. The events providing this Ca$^{2+}$-dependent type of cell behavior were studied in detail for the 1960s−1970s [16, 17].

At present, several types of the electro-gated channels have been discovered on the paramecium cell surface; they differed not only by pharmacological and physiological properties, but also by ion specificity and structure of gating mechanisms. They involve two channel types activated by depolarization and hyperpolarization, as well as Ca$^{2+}$-dependent potential-gated Mg$^{2+}$-, K$^{+}$-, and Na$^{+}$-channels [2, 9, 18, 19]. These channels have electrophysiological characteristics resembling those revealed in vertebrate muscles and nerve cells.

A characteristic feature of calcium channels in excitatory membranes of eukaryote cells is their capability for activation under effect of changes of the transmembrane electrical field. In studying calcium currents in somatic and presynaptic neuron membranes of some Metazoa, data have been obtained of heterogeneity of the Ca(v)-channel population with respect to their activation characteristics. These channels are distinguished both by their kinetic parameters and by sensitivity to inhibitors. The infusoria *P. caudatum* and *P. tetraurelia* also were found to have Ca(v)-channels differing by chemical-pharmacological properties [20]. Moreover, the evidence has been obtained for intraspecies heterogeneity in the channel population in paramecium cells [9]. It was established that, like vertebrate electrically excitable cells, conductivity of calcium channels in infusoria is reduced after replacing extracellular Ca$^{2+}$ with La$^{3+}$ or Cd$^{2+}$ ions. At the same time, use of organic blockers of vertebrate cell calcium channels, such as verapamil, D-600, and nifedepine turned out to be of low effect [2]. Other authors also have reported a low selectivity of organic calcium channel inhibitors affecting the infusorian channels [4]. Exceptions are neurotropic blockers used at high

(millimolar) concentrations as well as inhibitors of calmodulin [18, 21, 22]. Functional tests used for estimation of channel activity *in vitro*, have allowed determining selective conductivity of calcium channels in *P. caudatum*. Conductivity of two types of Ca(v)-channels in the media containing Ca$^{2+}$, Ba$^{2+}$, Mg$^{2+}$, and monovalent cations was estimated using the technique of reconstruction of ion channels in lipid bilayers. The highest conductivity of the Ca(v)-channels was found in the Ca$^{2+}$-containing medium [23].

An important criterion for properties of ion channels is their inactivation mechanism. Already the first measurements of calcium currents in paramecium cells under conditions of reliable turn off of output currents have shown that the time course of their inactivation is essentially similar to that of calcium currents in Metazoa. During a long depolarization the calcium current in infusorium cells is exponentially reduced; however, this reduction develops relatively slow (the time constant is 1 ms) till the value that does not exceed 1 Pa. Direct registration of Ca$^{2+}$ input into the cells in the process of PM depolarization showed the inactivation of testing current in paramecium cells to be accompanied by a simultaneous dose-dependent increase of [Ca$^{2+}$]$_i$. Like in Metazoa cells, an intracellular administration of EDTA led to a decrease of the calcium current [24]. The obtained data unequivocally indicate that the inactivation of calcium currents in the studied objects is caused by the input calcium current induced by the previous membrane depolarization (the "current-dependent inactivation" unlike the "potential-dependent inactivation" for other ion channels), but not by changes themselves of the membrane potential. The mechanism of block of calcium channels by intracellular Ca$^{2+}$ ions in the lower eukaryote cells, like in vertebrate cells, appears to be due to participation of the Ca$^{2+}$−CaM complex in binding with the channel [4].

Also of interest are the studies focused on the role of abiotic cations (Ba$^{2+}$ and Sr$^{2+}$) passing via calcium channels. These ions have been established to change kinetics of the Ca(v)-channel activation and inactivation processes in paramecia; however, the exact mechanism of their inhibitory effect on Ca(v)-channels is not yet clear [25].

Methods of molecular cloning and mutagenesis

are used more and more often for studying structure, regulation, and properties of calcium channels. Interesting studies have been carried out on *pawn* and *CNRs* strains of mutant *P. tetraaurelia* cells negative for several functions of the Ca(v)-channels. Using these mutants, it was proven that defects of the transmembrane calcium signal propagation (the absence of input calcium current; no action potential-induced $Ca^{2+}$ input into cell) led to disturbance of cell locomotion and motility [26].

The *pawn* and *CNRs* mutants have become widely used in experiments on separation of membrane currents [20], isolation of depolarizing mechanoreceptor potential [27], monitoring of the $Ca^{2+}$ and $Ba^{2+}$ influx via $Ca^{2+}$-channels *in vivo* [28], and in studying channel properties of vesicles [29, 30].

Up to now it has remained unknown which particular genes encode the calcium channel structure in paramecia. Good candidates for the role of model organisms to study structural properties of $Ca^{2+}$-channels are the so-called "dancer mutants" of *P. tetraaurelia*. Mutation of *Dn* gene has been established to cause a pronounced change of characteristics of calcium current in paramecium cells. Unlike the wild type of cells that generate leveling action potentials in response to depolarizing stimuli, the dancer mutants are more excitable and evoke action potential on the "all-or-nothing" principle [31].

Little is known at present about the structure of Ca(v)-channels not only in infusoria but also in other protist representatives. There are data that in myxomycetes of *Dictyostelium discoideum* the potential-gated $Ca^{2+}$ channels are formed by annexin VII that is a cytosol $Ca^{2+}$-binding protein [32].

Data about the Ca(v)-channels in cells of other lower eukaryotes are fragmentary and so far cannot yet be systematized.

*Receptor-gated $Ca^{2+}$-channels.* This group consists of the Ca(v)-channels that are activated exclusively by the receptor-mediated mechanism rather than as a result of plasma membrane depolarization. This class can include both true $Ca^{2+}$-channels and low-selective cation channels that are permeable first of all to monovalent cations, but also are able to transport $Ca^{2+}$ and thereby to control its intracellular activity.

The group of the true $Ca^{2+}$-channels includes the channels whose receptor agonist either performs the function of a channel or directly interacts with the channel structure. The prototype of these channels was the mammalian nicotine cholinoreceptor itself is directly a nonselective cation channel. The nicotine cholinoreceptor channel is permeable to $Ca^{2+}$, but under physiological conditions it transports predominantly $Na^+$ and $K^+$. Its selectivity to monovalent cations is 3—5 times as great as to $Ca^{2+}$.

Results of study of the mechanisms involved to $Ca^{2+}$-signaling of the hemoflagellate *Trypanosoma cruzi* suggests that the receptor-like membrane structures of these parasitic protozoa can serve evolutionary predecessors of the mammalian nicotine receptor. This possibility is indicated by studies of pharmacological properties of these structures showing sensitivity to acetylcholine and its derivates [33]. The authors have shown that the membrane of *T. cruzi* binds actively both antagonists and agonists of the acetylcholine receptor. Moreover, it was demonstrated that in *T. cruzi* epimastigotes, nicotine at a concentration of $1 \times 10^{-5}$ M induced a rise of $[Ca^{2+}]_i$ that did not depend on its external concentration, which suggested nicotine to stimulate mobilization of $Ca^{2+}$ from intracellular stores, such as endoplasmic reticulum (ER) and acidocalcisomes [33].

The mechanism of receptor-controlled intracellular $Ca^{2+}$ mobilization implies the presence in eukaryote cells of special structures depending on $Ca^{2+}$. Indeed, in mammalian cells, many proteins were revealed, their function and activity being regulated by $[Ca^{2+}]_i$. These proteins include, in particular, some protein kinases taking part in transmission of proliferative and chemotactic signals. These proteins are components of signaling pathways, in which $Ca^{2+}$ plays the role of second messenger.

The $Ca^{2+}$-messenger system in mammalian cells is activated by several ways including stimulation of the phosphoinositide signaling pathway [34, 35]. As a result of such activation the intracellular $Ca^{2+}$ mobilization occurs and local calcium signal is formed.

In unicellular microorganisms, pathways of $Ca^{2+}$ mobilization by activation of the G-protein-binding receptors and phosphoinositide system were also revealed [36]. This activation appears to

lead to stimulation of two independent mechanisms of Ca$^{2+}$ mobilization, one of them related to the external Ca$^{2+}$ input, the other coupled to release of intracellular Ca$^{2+}$ via channels activated by second messengers. In 1984, Bumann and co-authors were the first to establish in the lower eukaryote cells the fact of the agonist-dependent Ca$^{2+}$ influx associated with activation of serpentine receptors [37]. The authors have shown that the 5-min activation of receptors of the *D. discoideum* cell surface by chemoattractants (folic acid and cAMP) induces influx of external Ca$^{2+}$ into the myxomycete cells. To further study mechanisms of the receptor-controlled Ca$^{2+}$ influx in microorganisms, peculiarities of Ca$^{2+}$ transport in cells at various stages of their life cycle [38]. It was established that the calcium responses to folate and cAMP were regulated in different ways: in the first case, Ca$^{2+}$ influx occurred in vegetatively growing cells, while in the second case, in cells starting their differentiation. Other characteristics of these Ca$^{2+}$-importing systems turned out to be similar. The both systems had identical kinetic properties, had a high specificity for Ca$^{2+}$, and were inhibited by ruthenium red and sodium azide as well as by carbonyl cyanide-*m*-chlorophenylhydrazone. On the basis of data obtained by the authors it may be suggested that different chemoreceptors of *D. discoideum* stimulate the same pathway of the agonist-dependent influx of external Ca$^{2+}$ into cells. However, genetic studies performed on *D. discoideum* mutant cells defected for $G_{\alpha 1}$ and $G_{\alpha 2r}$ subunits have shown that the Ca$^{2+}$ influx into cells, although is related to activation of serpentine-type receptors, but is not regulated by heterotrimeric G-proteins [38].

Evidence for participation of G-proteins in the receptor-dependent [Ca$^{2+}$]$_i$ increase in *Dictyostelium discoideum* was obtained when using a platelet activating factor (PAF). It turned out that the phospholipid compound (1-*o*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a modulator of the wide-spectrum of second messengers in the higher eukaryote cells, could also induce Ca$^{2+}$ influx into the *D. discoideum* cells [39]. PAF is known to act through activation of G-proteins associated with membrane receptors; therefore, strains of mutant cells negative for $G_\beta$ and $G_{\alpha 2}$ subunits of the heterotrimeric G-protein were used in study-

ing mechanisms of action of this compound on unicellular fungi. It was shown that the calcium response to PAF was lower in modulator in the g$\beta$(−) cells, than in the wild type cells, and was absent completely in the cells $G_{\alpha 2}$(−); from this, the authors concluded that G-proteins participated in the mechanism of the receptor-dependent Ca$^{2+}$ influx into the *D. discoideum* cells. Inhibitor analysis with use of blockers of InsP$_3$ and inhibitors of phospholipases C and A (PLC and PLA$_2$) enabled the conclusion to be made that the PAF-induced Ca$^{2+}$ influx into the *Dictyostelium discoideum* cells was due to PLC activation and InsP$_3$ production and was independent of the presence of endo- and exogenous cAMP [39, 40].

In cells of the closely related fungi *Candida albicans*, two pathways of Ca$^{2+}$ mobilization were revealed, one of them induced by InsP$_3$, the other by internal positive potential. These pathways differ by the amount of released Ca$^{2+}$, by the nature of the calcium response, and by pharmacological characteristics [41]. A similar InsP$_3$-dependent pathway of Ca$^{2+}$ mobilization from vacuoles was revealed in the yeasts *Neurospora crassa* and *Saccharomyces cerevisae* [42, 43]. Specificity of the Ca$^{2+}$ release from intracellular stores implies that the calcium response in fungi is mediated through a receptor of certain type that resembles InsP$_3$R of the higher eukaryote cells. The presence of InsP$_3$R in fungi is indicated by fluctuations in the intracellular InsP$_3$ level throughout cell cycle of *Physarum polycephalum*, these fluctuations correlating with changes in kinetics of $^{45}$Ca$^{2+}$ exchange [44]. However, in *Candida albicans* and *Neurospora crassa* cells, use of inhibitor analysis that is widely applied to detect InsP$_3$R in vertebrate cells proved to be of little effectiveness [41, 42], which might reflect differences in the InsP$_3$R structure between fungi and vertebrates. On the other hand, there are genetic and biochemical data in favor of the existence of homology in the InsP$_3$R structure in cells of vertebrates and the lower eukaryotes [45].

The carbachol-induced receptor-dependent Ca$^{2+}$ release from intracellular stores was revealed in protists *T. cruzi* [46]. A two-phase increase of the InsP$_3$ level preceded the intracellular Ca$^{2+}$ mobilization. It was fast and short-term at the early phase of stimulation, with a maximum after 1 min and return to the basal level by 6 min. At the

second phase, the $InsP_3$ level rose at the 10–12th min, while decreased at the 20th min after the cell stimulation. Like in unicellular fungal cells, pretreatment of tryponasomes with a PLC inhibitor (U73122, 10 μM) led to complete inhibition of $InsP_3$ synthesis, but, unlike fungi, without appreciable changes in the dynamics of $[Ca^{2+}]_i$ changes. The initial phase of the $InsP_3$ metabolic rise also was absent in the cells incubated with activator of protein kinase C (PKC), phorbol-12-miristate-13-acetate, which can indicate the presence in this microorganism of a complex agonist-dependent mechanism of $Ca^{2+}$ mobilization involving a wide range of physiological modulators, like in cells of the higher eukaryotes. The complex mechanism of $Ca^{2+}$ mobilization with participation of $InsP_3$ as a second messenger has also been reveled in *Euglena gracilis* [47]. The receptor-dependent $Ca^{2+}$ mobilization coupled with activation of PLC and an increase of the $InsP_3$ level was found in cells of the ameba *Entamoeba histolytica*. It is to be noted that stimulation of membrane receptors by fibronectin in these microorganisms leads not only to intracellular, but also to extracellular $Ca^{2+}$ mobilization [48].

Interesting data have been obtained about the role of arachidonic acid, an important phospholipid messenger in mammals, in regulation of extracellular $Ca^{2+}$ influx and intracellular $Ca^{2+}$ release through channels of PM and acidocalcisome, respectively, in hemoflagellate *T. cruzi* [49]. In mammalian cells, the principal sources of arachidonic acid are phosphoinositides and diacylglycerol formed after their hydrolysis. In protists, most of free arachidonic acid seems to be formed during hydrolysis of intracellular phospholipid sources including diacylglyceropyrophosphate (DGPP) [50].

Activation of receptors coupled with PLC in cells of Metazoa initiates a signal cascade leading to simultaneous formation of several second messengers that are activators of $Ca^{2+}$ mobilization from intracellular stores [35]. Metabolite of nicotinamide dinucleotide, cyclic adenosine diphosphate ribose (cADPR), is considered as a trigger producing the intracellular $Ca^{2+}$ mobilization independent of products of phosphoinositide metabolism [51]. In mammalian cells, the cADPR messenger system is suggested to act through ryanodine receptors. It has turned out to be unexpected that cADPR functions already in the lower eukaryote cells [47]. However, its physiological role in protists is unclear.

Thus, the lower eukaryote cells contain not only structural components necessary for formation of the calcium signal (as in eukaryote cells), but also the mechanisms of agonist-induced influx and intracellular $Ca^{2+}$ mobilization, which served as evolutionary predecessors of the mechanisms in the higher eukaryote cells.

## MODULATION OF CALCIUM SIGNAL

Modulation of calcium signal occurs by a mechanism providing regulation of $[Ca^{2+}]_i$. The mechanism involves $Ca^{2+}$ transport elements (calcium storing organelles and $Ca^{2+}$ transporters) as well as the receptor $Ca^{2+}$-binding proteins (CaBPs). In this section, we will consider structure and functions of CaBPs, as they play the key role in modulation of calcium signal in eukaryote cells.

The $Ca^{2+}$-binding proteins are primary $Ca^{2+}$ receptors and are necessary for calcium to act as the intracellular second messenger. As a result of this interaction, CaBPs change their conformation structure and acquire the capability for activation of several enzymes and regulatory proteins to affect thereby dynamics of numerous intracellular processes. In the lower eukaryote cells, CaBPs participate in regulation of the cell cycle and affect activity of various enzymes and signal proteins, transmembrane $Ca^{2+}$ ion transport, and the state of cytoskeleton or play a role of intracellular $Ca^{2+}$-buffer (calsequestrin). Most of CaBPs revealed in protists belong to proteins of the "EF-hand" superfamily.

Proteins of this superfamily have specific $Ca^{2+}$-binding domains. The central link of such domains is a $Ca^{2+}$-binding loop composed of 12 amino acid residues, a part of them containing oxygen atoms in their side chain. On either side of the $Ca^{2+}$-binding loop, α-spiral areas are located; therefore, structure of the $Ca^{2+}$-binding domain has the form of spiral–loop–spiral. This domain, due to its structure with the regular arrangement of the oxygen-containing amino acid residues, provides the highly effective and specific $Ca^{2+}$ binding. In the higher eukaryote cells, CaBPs containing from two

to six Ca$^{2+}$ binding domains were found [52].

It was long believed that CaBPs existed only in the highly organized animal and human cells; however, proteins with a high affinity for Ca$^{2+}$ ions, including CaM-like proteins, were also detected in the lower eukaryotes and even bacteria. Moreover, it was shown that some of them revealed in prokaryote cells included two and more sites similar structurally with the EF-hand domain [53]. Nevertheless, these proteins differ markedly from CaBPs of Metazoa [1].

Quite a few CaBPs belonging to the protein superfamily that has the EF-hand domain have been revealed in the lower eukaryotes [54]. Structurally, they are closer to the calcium- binding proteins of multicellular organisms than to CaM-like proteins of bacteria. Information about properties and functions of CaBPs in the lower eukaryote cells is fragmentary. Described in the greatest detail are biochemical properties of the CaM-like proteins revealed in cells of various taxonomic protistic groups.

The existence of CaBPs in the lower eukaryotes was first reported in the early 1970s. Acidic low-molecular CaBPs with a high affinity for Ca$^{2+}$ binding were extracted from cells of infusoria *Zoothamnium geniculatum* [55]. The authors have established these proteins (spasmins) to bind Ca$^{2+}$ at concentrations ranging from $10^{-6}$ to $10^{-8}$ M and to thereby provide the contractile function. Later, proteins (mol. weights of 16, 18, and 22 kDa) with similar properties were isolated from cells of infusoria *Carchesium colypinum* [56]. In the immuno-precipitation reaction, antibodies to these CaBPs cross reacted with spasmins of the infusorian genuses Stentor, Spirostomum, and Blefarisma, but not with mammalian calmodulin and troponin C [57]. Subsequently, CaBPs were detected in the flagellate *Euglena gracilis*, ameba *Amoeba proteus*, and myxomycete *Phusarum polycephalum* [58].

The high affinity CaBPs of mol. mass 24 kDa (K$_d$ < 50 μM Ca$^{2+}$) with the low binding capacity for Ca$^{2+}$ ions (less than 2 moles Ca$^{2+}$ per 1 mole of protein) were revealed in hemoflaggelates *T. cruzi*. These proteins were shown to participate in processes providing mobility of the parasite [59]. In the closely related species *Trypanosoma brucei*, the 22, 24, and 38 kDa CaBPs were found, with their biochemical and immunological properties differing from those of known proteins belonging to annexins and of proteins of the EF-hand superfamily. It is of interest that the 22 kDa CaBPs in the N-terminal region have a significant homology (58%) with that of the mammalian p21 tumor protein [60]. The flaggelate p27 protein of the protozoan parasite Chlamydomonas has three domains, two of which are Ca$^{2+}$ binding EF-hand domains. The target of this protein is nucleoside diphosphate kinase (NDK) requiring GTP for its activation, which indicates involvement of p27 protein in the GTP-mediated signal pathway providing mobility of the parasite [61].

In unicellular eukaryotes, examples of proteins containing three or four cation binding centers are granins, TCBP-23 and TCBP-25 proteins, centrin, and caltractin, as well as calmodulins and CaM-like proteins. Group of proteins with three calcium binding domains includes granin 1 and granin 2 isolated from cytoplasmic granules of the protozoan parasite *Entamoeba histolytica*. These proteins are involved *in vivo* in processes of endo- and phagocytosis [62].

Among the proteins containing four EF-hand domains in their structure, the simplest is the low-molecular EhCaBP protein also isolated from *Entamoeba histolytica* cells [63]. This protein characterized by a low structural homology (30%) relative to other known CaBPs, including CaM, contains two globular domains, each consisting of two pairs of the spiral−loop−spiral sites similar topologically with those in the mammalian CaM molecule. This group of CaBPs also includes highly specialized centrin and spasmin-like proteins participating in formation of the microfilament system in protists and lower fungus cells [56, 57, 64−66]. It was shown on the example of the protozoan parasite *Leishmania donovani* that centrin took part in processes of centrosome duplication and segregation, which indicates a functional role of this protein in control of cell growth [67]. The most divergent protein in the centrin family is a centrin-like DdCrp protein of the fungus *Dictyostelium discoideum*. This protein has only two EF-domains, while its primary structure contains all conservative sequences typical of centrins [66]. It also differs functionally from vertebrate centrins. Using immunofluorescence analysis and confocal microscopy, it was established that DdCrp protein

was located near the nucleus and inside the nucleus and is associated with the centrisome by surrounding its central part as a specific fluorescent corona. In the process of prometaphase, $Ca^{2+}$ dissociation from the protein occurs and coronal fluorescence disappears, which indicates participation of DdCrp in the process of mitosis.

In the lower eukaryotes, the best studied among calcium-binding proteins containing four EF-hand domains is calmodulin (CaM). In protists, like in Metazoa cells, CaM is a regulator of diverse cell functions and acts preferentially through modulation of different enzyme activities, including adenylyl and guanylyl cyclases, phosphodiesterases, calcineurin, $Ca^{2+}$-ATPase, phospholipase $A_2$, and some others [68−72].

The widespread of CaM in unicellular organisms seems to be due to that this protein, as a mediator of calcium signaling, is needed for various $Ca^{2+}$-dependent functions. It has been established that the CaM−$Ca^{2+}$ complex activates cell mobility in protists, regulates cell cycle, and initiates cytokinesis, as well as plays an important role in viability and intracellular invasive process, i.e., essential life cycle components in parasitic protozoa.

A comparison of CaM of the higher and lower eukaryote cells has shown that mammalian CaM by its primary structure, physical and chemical properties is the closest to the CaM of lower fungi [73]. Thus, for instance, the CaM composed of 149 amino acids from dividing cells of the yeast *Schizosaccharomyces pombe* has a 74% identity in its amino acid sequences with CaM from bovine brain [74], while CaM (mol. weight of 40 kDa) isolated from cells of the fungus *Physarum polycephalum* has a 88% identity with that from bovine brain [75]. A high homology of amino acid sequences in CaM of the lower fungi has been reported in the C-terminal part, whereas the N-terminal region lacks calmodulin sequences. The primary structural similarity of calmodulins isolated from mycelium of the lower fungi *Agaricus campestris* and *Coprinus lagopus* and the bovine brain is the basis for orthological similarity of these enzymes and explains why kinase of the muscle myosin light chain is activated *in vitro* to the same extent by calmodulins from mammalian and fungal cells [73].

The identical properties were established for CaM-like protein found in cells of budding yeasts *Saccharomyces cerevisiae* [73]. On the example of the CaM-like protein isolated from cells of dividing yeasts *Schizosaccharomyces pombe* it was shown that this protein encoded by the *cam1* gene participated in regulation of processes of vegetative growth of microorganisms [74]. The Ca-like protein Ca1B was isolated from the mucous fungus *Dictyostelium discoideum*; this protein has properties similar with those of the mammalian antigen, as well as close amino acid sequences (50% identity) and the exon−intron structure of its encoding gene [75]. Maximal expression of the Ca1B protein was observed in the course of cell aggregation and in fungal spores, which might indicate a great importance of this protein for regulation of processes of cell differentiation and sporulation.

The presented examples of the regulatory CaM action on various intracellular processes in the lower eukaryotes indicate that protistic CaM can modulate activity of numerous $Ca^{2+}$-dependent enzymes and proteins-effectors. The question arises of how does this protein recognize and regulate activity of tens of different protein targets? It has turned out that the mechanisms of its effect on intracellular subunits of eukaryote microorganisms are basically the same as in the higher eukaryote cells. The basis for ligand-mediated modulation of activity by calmodulin consists of three processes: recognition of substrate on the principle of complementarity (the presence of amphiphilic α-spirals in proteins), activation of protein-target by phosphorylation-dephosphorylation, and, finely, inhibition of activity of the CaM-dependent enzymes as a result of $Ca^{2+}$ removal from the calcium-binding CaM sites and subsequent dissociation of CaM from a complex with a regulatory site of the enzyme [54, 77, 78].

The so-called CaM-like domain protein kinases (CDPKs) described in the works [79, 80] play a special role in regulation of the $Ca^{2+}$-dependent contractile processes in unicellular eukaryotes. Unlike kinases belonging to CaMK and PKC families predominant in mammalian cells, activation of the CDPKs requires $Ca^{2+}$ ions, but not phospholipids and calmodulin [80, 81]. At present, ancestor CDPK gene is believed to be originated from fusion of genes of protein kinase and calmodulin by recombination of ancestral introns [80].

The structurally most complex calcium-binding

proteins contain six Ca$^{2+}$-binding EF-hand domains. In mammals, this protein group includes calbindin isolated from intestinal cells and calretinin found in neurons. Their functions have remained so far poorly understood. The CaBP also containing six canonic Ca$^{2+}$-binding EF-hand domains was found in parasitic protozoa *Trichomonas suis* [82]. Function of this protein is suggested to be connected with regulation of the parasite flaggelar mobility.

Among other Ca$^{2+}$-binding proteins, annexins were detected in unicellular eukaryotes [83]. In paramecium, these Ca$^{2+}$-dependent phospholipid-binding proteins are selectively recognized by antibody against the common annexin sequences and are located in sites of anchoring trichocysts and cytoprocts [84].

Apart from annexins, proteins belonging to a new group of Ca$^{2+}$-dependent phospholipid-binding proteins, copines, were revealed in Paramecium [85]. These proteins contain Ca$^{2+}$-binding domains composed approximately of 120 amino acids. The functional role and the exact location of copines in infusorian cells are so far unknown.

Calnexins, proteins of the calreticulin family, were found in protozoa *Trychomonas suis*, *Leishmania major*, and *Euglena gracilis* [86−88], as well as in fungi *Dictyostelium discoideum* [89]. Their calcium-depositing function in protozoan cells is not yet quite clear.

Thus, the mechanism of calcium signal modulation by CaBPs, which operates in prokaryote cells, has got its further development in protists and, by acquiring new regulatory elements of input signal in the course of evolution, has become the structural-functional basis for formation of such mechanism in the higher eukaryote cells.

## MECHANISM OF CALCIUM SIGNAL EXTINCTION

A peculiarity of the calcium signaling in eukaryote cells is a short-term local calcium signal, as the long-term [Ca$^{2+}$]$_i$ increase that is not associated with formation of directed calcium wave leads to an intracellular pathology and cell death [90]. Therefore, the mechanism of extinguishment of the calcium signal is the central component of the calcium signal system. The membrane proteins-carriers included in the regulatory-transport system providing the [Ca$^{2+}$]$_i$ recovery to the level characteristic of the quiet cell take part in the calcium signal extinguishment.

Like metazoan cells, unicellular eukaryotes have effective mechanisms of extinguishment of calcium signal with the aid of protein carriers. The protozoan membrane contains several carriers responsible for Ca$^{2+}$ release from a cell. One of them has the highest affinity for Ca$^{2+}$ and has been revealed in most studied protists species. The carrier represents calcium ATPase, an enzyme that removes Ca$^{2+}$ from cell against a pronounced concentration gradient existing on the plasma membrane. The calmodulin-dependent plasma membrane Ca$^{2+}$-ATPase found in cells of infusoria *Paramecium tetraurelia* is the closest to the mammalian Ca$^{2+}$-ATPase. Like in cells of the higher eukaryotes, Ca$^{2+}$-ATPase reacts to even a slight increase of the intracellular calcium concentration [91, 92]. The calcineurin-dependent Ca$^{2+}$-ATPase called PAT1 has been revealed in PM of the myxomycetes *Dictyostelium discoideum* and in the membrane of the contractile vacuole [93]. In experiments with use of mutants deficient for PAT1 and with disturbed function of the contractile vacuole, the PAT1 and the contractile vacuole have been established to be components of mechanisms for Ca$^{2+}$ removal from *D. discoideum* cells and to serve for maintenance of Ca$^{2+}$ homeostasis, especially under conditions of Ca$^{2+}$-stress [93]. The vacuole membrane of budding yeasts *Saccharomyces cerevisae* contains Ca$^{2+}$-ATPase Pmc1p with a high affinity for Ca$^{2+}$, which is encoded by *pmc1* gene and has a 40% homology to the mammalian PM Ca$^{2+}$-ATPase. The Pmc1p does not contain the CaM-binding domain in the C-terminal, but in all other aspects it is the typical Ca$^{2+}$-pump and, like the Ca$^{2+}$-ATPases of metazoan plasma membranes, performs both the high-affinity catalysis and the ATP-dependent transmembrane Ca$^{2+}$ transport [94].

Another protein-carrier that responses to a sharp increasing of [Ca$^{2+}$]$_i$ in eukaryote cells is a transport protein exchanging calcium for sodium. Among the higher eukaryote cells, the highest content of this protein is present in excitable cells (nerve and muscle) that experience manifold sharp elevation of Ca$^{2+}$ concentration in response to

stimulation. In the lower eukaryotes, such way of extinguishment of $Ca^{2+}$ signal does not appear to be widely spread, whereas it is present in mobile cells of the freely living infusoria *Euplotes crassus* [95].

The mechanism of the $Ca^{2+}$-signal extinguishment includes elements of a system for maintenance of calcium homeostasis, which are already present in prokaryote cells. These elements are represented by the $Ca^{2+}/H^+$-exchanger that stimulates $Ca^{2+}$ release from bacterial cells. The $Ca^{2+},H^+$-ATPase was found in eukaryotic microorganisms of various taxonomic groups, including protozoa and lower fungi [96–99]. Unlike $Ca^{2+}/H^+$-exchanger in bacteria, in protists this transporter rather provides the $Ca^{2+}$-depositing function, than function of $Ca^{2+}$ export.

Other mechanisms of $Ca^{2+}$ removal from cells of lower eukaryotes also exist, but their contribution to the $Ca^{2+}$-signal extinguishment is less significant s compared with the above- mentioned mechanisms.

## CONCLUSION

Calcium is one of universal regulators of intracellular processes occurring not only in cells of higher organisms, but also in unicellular eukaryotes. Analysis of the data accumulated by the present time indicates that the main direction of both molecular and functional evolution of mechanisms of intracellular signaling in eukaryotes is associated with development of the calcium messenger system that is revealed practically in all current representatives of ancient species of unicellular eukaryote microorganisms (unicellular algae, protists, myxomycetes, and yeasts). The initial basis for formation of the calcium signal mechanisms in protists were elements of the $Ca^{2+}$-transport system and system of maintenance of calcium homeostasis dating back already to prokaryotes, including $Ca^{2+}$-channels, transmembrane $Ca^{2+}$-carriers, and primitive forms of $Ca^{2+}$-binding proteins.

The structural-molecular organization of the system of transmission of triggering information from the cell surface to intracellular targets was modified in the course of progressive evolution. Apart from the $Ca^{2+}$-sensitive sensor molecules existing already in bacteria, the unicellular eukary-

otes acquired highly specialized mechanisms of potential- and receptor-mediated transduction of the trigger signal to a local $Ca^{2+}$ signal and its subsequent translation to post-receptor structures. In the lower eukaryotes, for the first time in evolution, the system of modulation of calcium homeostasis appears with the aid of highly specialized $Ca^{2+}$-binding proteins. To provide this function, primitive mechanisms of $Ca^{2+}$ mobilization from the intracellular resources are formed in eukaryotic microorganisms. Some of these mechanisms obtained their evolutionary development in cells of the higher eukaryotes.

Like in metazoan cells, the mechanisms of $Ca^{2+}$-signaling in the lower eukaryotes seem to be based on processes of formation and directed propagation of the wave-like increase of the intracellular $Ca^{2+}$ concentration (the calcium wave) and possibly local $[Ca^{2+}]_i$ oscillations affecting development of programmed cell processes. However, information on this issue is not yet sufficient. The fact that calcium oscillations accompany the process of conjugation in infusoria and of differentiation in myxomycetes can be an argument in favor of this suggestion.

At present, the total structural-functional organization of $Ca^{2+}$-signaling mechanisms has been described sufficiently well in the lower eukaryotes, in particular, mechanisms have been studied of $Ca^{2+}$ input into cells, functional characteristics and ways of regulation of $Ca^{2+}$-channels have been described, various mechanisms of $Ca^{2+}$ mobilization from intracellular stores have been studied, and systems of emergency and long-term $Ca^{2+}$ removal from cells have been established. However, protein kinase pathways of signal transduction with participation of the $Ca^{2+}$-messenger system in the lower eukaryote cells are still far from their complete understanding.

## ACKNOWLEDGMENTS

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