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Review

Macromolecular Crowding: a Hidden Link **Between Cell Volume and Everything Else**

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Key Words

Cell volume regulation • Cell volume sensing • Macromolecular crowding • Apoptosis

Abstract

High density of intracellular macromolecules creates a special condition known as macromolecular crowding (MC). One well-established consequence of MC is that only a slight change in the concentration of macromolecules (e.g., proteins) results in a shift of chemical equilibria towards the formation of macromolecular complexes and oligomers. This suggests a physiological mechanism of converting cell density changes into cellular responses. In this review, we start by providing a general overview of MC; then we examine the available experimental evidence that MC may act as a direct signaling factor in several types of cellular activities: mechano- and osmosensing, cell volume recovery in anisosmotic solutions, and apoptotic shrinkage. The latter phenomenon is analyzed in particular detail, as persistent shrinkage is known both to cause apoptosis and to occur during apoptosis resulting from other stimuli. We point to specific apoptotic reactions that involve formation of macromolecular complexes and, therefore, may provide a link between shrinkage and downstream responses.

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Introduction

Macromolecular crowding (MC) and cell volume (CV) are closely related subjects that happen to be pursued by separate groups of investigators. MC research mostly focuses on in vitro models and on theory, with only occasional incursions into live cell research. CV literature, with some exceptions [1-4], pays little attention to the MC effects or, at best, mentions them in passing. But MC is not an exotic or a controversial subject; it often appears in the more traditional guise of intracellular water content and reveals itself under the optical microscope as a refractive index. Thus, the discussion of MC could easily be conducted using a different and perhaps more familiar vocabulary; the reason we still prefer the term "macromolecular crowding" is that this keyword takes us directly to some of its most universal effects.

MC can be operationally defined as the concentration of macromolecules in mass/ volume units. The main intracellular macromolecules are proteins (making up 60-75% of

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dry mass in mammalian cells) and nucleic acids (5-10% of dry mass) [5-7] with the average protein-to-DNA mass ratio of 3.35 [8]. Because of the abundance of proteins and following the convention adopted in the MC literature, for the rest of the paper we will be referring to macromolecules as "proteins". However, the classical MC effects are supposed to be largely independent of the nature of "crowder" (which is not always observed in reality), and the typical *in vitro* crowding experiment often uses inert polymers (dextran, Ficoll, or polyethylene glycol) to mimic the environment inside the cell [9]. The next approximation to nature can be achieved by encapsulating the reagents and crowders in liposomes [10-12]. In this paper, we will look at the crowding effects from the CV regulation perspective, including their possible roles in the regulatory volume responses, volume sensing, and apoptosis. The paper will be mostly focused on mammalian cells; discussions of MC in yeast, bacteria, and plants can be found elsewhere [13-16].

A partial list of MC values is given in Table 1. Since the average density of proteins in a hydrated state is close to 1.4 g/ml [17], its concentration can be converted into volume fraction W occupied by water as W = 1 - 0.70C [6]. Thus, 85% of the volume of a typical healthy eukaryotic cell is occupied by water, and the remaining 15% is taken up by proteins.

Quantification of macromolecular crowding in living cells

MC can manifest itself through increased osmotic pressure, conformational changes, and increased viscosity; various methods have been developed to measure each of these characteristics [32, 33]. But of course, the most direct aspect of MC is protein concentration (or the complementary quantity of the fraction of the total volume occupied by water).

Species	Cell type	C (g/ml)	Method	Reference
Human	HeLa adherent	0 20-0 21	TIE/TTD	[18]
mannan	nela, aanerene		TIE/TTD	[19]
Human	HeLa, suspension	0.24	TIE/TTD	[19]
		0.25	Hilbert phase	[20]
Human	Prostate cancer, DU145	0.19	TIE/TTD	[18]
Human	lymphocytos	0.10	Density	[21]
IIuiiiaii	lymphocytes	0.19	Microfluidic mass sensor	[22]
Human	Spleen cells	0.19	Density	[23]
Human	Smooth muscle	0.12	TIE/confocal	[24]
Human	Pancreatic tumor	0.22	Digital holography	[25]
		0.19	TIE/TTD	[18]
Dog	MDCK	0.19	TIE/TTD	[19]
		0.26	Interferometry	[26]
Mouse	3T3 fibroblasts	0.18	TIE/TTD	This work
Mouse	Cortical neurons	0.18	TIE/TTD	This work
Mouse	Neurons	0.20-0.21	Digital holography	[27]
Rat	Kidney, RK3E	0.09	Optical coherence	[28]
Frog	Various	0.15-0.25	Weight	[29]
Yeast		0.20	Viscosimetry	[30]
E. coli		0.3-0.4	Weight	[31]

Table 1. A partial list of the reported values of the average intracellular protein concentration. When the refractive index was measured, it was converted to dry mass concentration as $C = (n_{cell} - n_{medium})/0.19$, using the number for n_{medium} provided by the authors. Cell density d_{cell} was converted to C as $C = 2.5(d_{cell} - 1)$. When water mass fraction F_w was determined by drying, we used the formula $C = (1 - F_w)/(1 + 0.4F_w)$ [6]. MC in mouse fibroblasts and cultured cortical neurons were determined by the method of Mudrak et al. [18]

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Measurement techniques for intracellular protein concentration have been recently reviewed [6, 7]; more literature, in which MC is discussed in terms of refractive index [34], is available for technically inclined readers. Refractive index is closely related to protein concentration since the latter is the main reason for the stronger refraction of cells compared to water:

 $n_{cell} - n_{water} \approx 0.19 \cdot C(g/ml)$

We are not going to replicate the information already covered in the cited papers but would like to alert the reader to a common misunderstanding.

Quantitative phase imaging (QPI) provides a practical approach to MC measurement. However, the popular digital holographic microscopy only measures cell mass; thus, additional knowledge of CV is necessary to extract the cell-averaged protein concentration. The question then shifts to how to measure CV. A number of approaches are available, with various degrees of compatibility with QPI [6, 35]; our preferred QPI/CV combination is the transmission-through-dye (TTD) method for CV [36, 37] and transport-of-intensity equation (TIE) imaging for QPI [18, 38]; both can be readily realized on a standard optical transmission microscope and allow simultaneous fluorescence imaging.

The other point to be aware of is that quality control on some commercial QPI instruments is lacking. One simple way to test a QPI technique for consistency (if not for accuracy) is to image the same cell before and after adding a hypotonic or a hypertonic solution. A change in tonicity would cause a change in cell size and shape, but the cell mass reported by the instrument must be conserved [39]. It seems that not all digital holographic microscopes pass this test successfully.

While cell-averaged concentrations provide a single easily interpretable number, its obvious limitation is that it is just cell-averaged. Mitochondria, lysosomes, and nucleoli have a higher protein content than the cytosol or the nucleus [6], and the space immediately next to the membrane may experience a different extent of crowding (A. Minton, personal communication); even the bulk cytosol is highly heterogenous [40]. Thus, not all processes can be equally well captured by space-averaged measurements. Spatially resolved quantitative phase combined with high-resolution fluorescence microscopy may help extend the investigation to the organelle level [41, 42].

The other potential caveat is that marine and some other animals can accumulate large amounts of organic osmolytes, such as proline, betaine, urea, or glycerol [43, 44]. These compounds will significantly contribute to the refractive index but, due to their small size, may only slightly affect the typical MC phenomena, such as the tendency of proteins to aggregate.

Why MC may have biological effects

When the cellular composition is analyzed by atomic spectroscopy and is related to sample volume, one obtains concentration. However, chemical and biological reactions are driven not necessarily by concentrations C but by "effective concentrations", or thermodynamic activities A. It is the activities that are measured with electrodes or with chemical indicators; the activities determine reaction rates and are responsible for osmotic pressure.

Thermodynamic activity is related to concentration through the activity coefficient γ = A/C. The activity of non-interacting molecules (as occur in very dilute solutions) is equivalent to their concentration, in which case γ = 1. However, at higher concentrations, when particles or solutes interact with each other or with other solutes, γ may significantly deviate from unity. For example, γ = 0.82 for NaCl in a 0.05 M salt solution and γ = 0.68 in a 0.5 M solution (https://en.wikipedia.org/wiki/Thermodynamic_activity).

Thermodynamic activities of proteins are also affected by high concentrations. The activity is closely related to chemical potential, which includes enthalpic and entropic components; the latter depends on the available volume. Once the space becomes filled

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with large molecules, it becomes disproportionately difficult to fit another large molecule into the remaining free space. The result is a steep increase in the activity coefficient for large molecules when they are present at high concentrations [45-47]. For example, γ for hemoglobin at protein densities encountered in red blood cells approaches or even exceeds 100, and a 10% increase in hemoglobin density from 250 mg/ml to 275 mg/ml would increase its activity by two-fold [45]. (Notice that these numbers do not translate into the same factor for osmotic pressure because the linear relationship between osmotic pressure and concentration expressed by the Boyle-Van't Hoff law holds only for small concentrations; in a more general case, the relationship is logarithmic).

The increase in the activity coefficient of large molecules at a high density is referred to as macromolecular crowding or the volume exclusion effect. It has at least three important consequences [45, 48, 49]. First, it promotes the formation of macromolecular complexes and oligomers, including protein binding to membranes [50]; thus, in situations when cellular responses involve such reactions, they are likely to be enhanced by MC. Second, MC favors more compact protein conformations. Third, it affects reaction rates. But the latter is not easily predictable because, while higher protein density pushes molecules together once they are within the reaction distance, a slower diffusion may reduce the frequency of collisions and hence the reaction rate.

The determinants of MC

In the absence of cell growth or cell fragmentation, MC dynamics is inversely related to cell volume, and many of the facts known about CV regulation can be extended to MC. However, unlike CV, MC reflects cell composition. If N_p is the number of protein molecules in a cell and μ_p is their average molecular weight, then

$$MC = \frac{N_p \mu_p}{CV} \quad (1)$$

Intracellular osmolarity is set mostly by inorganic ions I and by small organic metabolites (which we designate as "amino acids", although they comprise various types of chemicals). In a balanced state, intracellular osmolarity must be equal to extracellular osmolarity E, resulting in the condition:

$$\frac{N_{aa}}{CV} + [I] = E \qquad (2)$$

For simplicity, we used here molar concentrations instead of activities, which should not affect the main conclusions. By expressing cell volume through this equation and substituting it into the expression for MC, we obtain:

$$MC = \frac{N_p \mu_p(E-[I])}{N_{aa}} \quad (3)$$

Thus, the preservation of MC would require the maintenance of a constant ratio between amino acids and proteins. If, for example, anabolism prevails, so that N_p increases and N_{aa} drops (or increases less than proportionately), MC is expected to increase. It is possible that the higher refractive index of cancerous cells and of normal cells during the G2/M phase [34] is related to this metabolic shift. A change in external osmolarity or membrane permeability for ions will also cause a change in MC due to water redistribution – in this sense, MC regulation is similar to CV regulation.

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Macromolecular crowding in action: a review of evidence

Stability and variability of MC

It is remarkable that, with very few exceptions (which may be due to technical issues), all healthy eukaryotic cells under stable conditions have a protein density close to 0.2 g/ml (Table 1). More data from the diffusion perspective, also showing the similarity between different cell types, can be found in [51]. What could be the reason for such consistency?

Although MC is ubiquitously present and forms the biochemical "background" on which all other biological responses are unfolding, its exact role (or roles) remains elusive. Persistent elevation of MC leads to apoptotic cell death [52], and this case will be discussed later in the paper. Apparently, the lowest macromolecular concentration in healthy cells, 70 mg/ml, has been reported in chondrocytes during bone growth [53]. Some freshwater mollusks have an extremely low blood osmolarity, down to 30 mOsm/kg [54], and it would be interesting to measure their macromolecular content. Other than that, life seems to resist a dilute state (except for temporary or pathological osmotic swelling), and one can only speculate on the reasons. For example, weak interactions between metabolically linked enzymes ("metabolons"; [55]) may become disrupted at low protein density. Maximization of metabolic rate is another possibility discussed in the literature [16, 56].

The only way to investigate the role of a factor is to have that factor specifically altered. The most direct way to alter MC is to expose cells to an anisosmotic solution. The list of responses elicited by osmolarity is long, including changes in DNA transcription, protein synthesis, membrane lipids, and organelles [57-59]. Cells continuously cultured in hypotonic media become smaller, and in hypertonic media they become larger [60-63]. Many of these responses may be, directly or indirectly, mediated by MC. Unfortunately, long-term changes in MC have not been reported in these studies, and it is possible that MC eventually returns to the initial values. Indeed, the possibility of MC homeostasis has been discussed theoretically [16] and later demonstrated experimentally. By exposing fission yeast to repeated osmotic shocks, Knapp et al. [64] managed to create an increased intracellular protein density; after a return to a stable osmolarity, the increased MC has dissipated toward the normal values over the course of several cell generations. Our lab has also observed a gradual restoration of MC in HeLa cells transferred to a hyperosmotic medium [18].

Changes in MC can also result from non-osmotic perturbations. Preventing mitosis while allowing cell growth leads to dilution of the cytoplasm and cell impairment on multiple levels [65]. However, in those experiments, the primary noxious factor was a reduced amount of DNA, and a decreased MC was rather a consequence. It would have been interesting if the authors tried to oppose cytoplasmic dilution by simultaneously increasing the external osmolarity to see which downstream effects are related to MC.

Generally, MC responds to adverse conditions and to numerous situations in which membrane permeability undergoes a change [66-70]; however, like in the example with mitotic arrest, there is usually not enough ready information to decide whether MC exerts a direct biological effect.

In the next few sections, we will examine several situations in which MC is likely to control cell behavior, including its own maintenance.

Cell responses to osmolarity: general considerations

CV maintenance in the face of variable external osmolarity has been a subject of voluminous literature. MC regulation is not identical to CV regulation but, as we hope to show, may be involved in the latter.

The concept of CV regulation applies mostly to rapid responses or to terminally differentiated cells that do not grow and divide. Following an abrupt exposure to an anisosmotic solution, cell volume often recovers, suggesting that cells somehow sense their volume. Other examples of volume "awareness" include cell division once a cell reaches a certain size and possibly osmosensing by bacteria, aquatic animals, and by specialized neurons in the hypothalamus. The nature of the primary CV sensor has not been discovered yet, and

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the literature is replete with hypotheses: membrane stretch, cytoskeletal rearrangements, integrins, pH, protein phosphorylation, calcium, other second messengers, and, occasionally, macromolecular crowding [59]. However, there is a fundamental difference between MC and other putative CV sensing mechanisms: whenever CV undergoes a change without a matching loss or gain in dry mass, a change in MC occurs with certainty, whereas other responses require special conditions. For example, membrane tension that is supposed to increase during hypoosmotic swelling may quickly relax due to the unfolding of membrane invaginations [2, 71, 72] and probably does not change at all during shrinkage. Sensing by the cytoskeleton is a possibility favored by many, but it implies the existence of rather elaborate structures that would always respond to swelling or shrinkage in a predictable manner [73]. The other factor that naturally and inevitably responds to a volume change is the concentration of intracellular ions and metabolites. As for the second messengers and other classical signaling factors, they are not expected to *directly* respond to the geometrical or mechanical factors resulting from shrinkage or swelling and would require an upstream mediator. Thus, we are left with four main candidates for the role of the primary volume sensor:

- (1) Intracellular concentrations
- (2) Membrane tension
- (3) Stress in the cytoskeleton
- (4) Macromolecular crowding

We will now consider cell responses to osmolarity in more detail.

Regulatory volume response

A rapid change in external osmolarity is frequently encountered by epithelial cells of the mouth and esophagus or by blood cells during passage through the peritubular capillaries of the kidney medulla. The fast rate of swelling or shrinkage does not allow enough time for adjustments in the amount of protein, and both CV and MC undergo an abrupt change. To mimic this situation in the laboratory, it is common to subject cells to a step change in osmolarity and follow their response immediately.

Many cell types respond to swelling or shrinkage by a rapid compensatory response known as regulatory volume decrease (RVD) or increase (RVI). The question we need to address is whether MC may act as the initial sensor of osmolarity-induced volume changes. Such a possibility has been discussed in at least three reviews dedicated specifically to this subject [1, 74, 75]. The idea has been noted by other authors but remained largely in the background.

Direct experimental evidence for MC as a signaling factor responsible for regulatory volume changes is indeed very limited at present, but a tentative case can be made. As has been well established, rapid RVI and RVD depend on the activation of monovalent ion channels followed by water redistribution. The return of hyposmotically swollen cells to their resting volume (RVD) is mediated by a net efflux of KCl, and the return of hyperosmotically shrunken cells (RVI) to their resting volume depends on a net efflux of NCl; the molecular nature of channels and transporters mediating volume recovery depends on the celltype [76].

Can any of these channels be regulated by MC? At least in some cases, the answer is positive. Colclasure and Parker demonstrated activation of K^+ -Cl⁻ cotransport by a decrease in MC [77] and of Na⁺-H⁺ by an increase in MC [78] in resealed erythrocyte ghosts. The proposed underlying mechanism assumes that the membrane channel responsible for the RVD can exist in two states, inactive phosphorylated and active dephosphorylated [79]. Swelling (a decrease in MC) induces dissociation of the kinase from the channel, resulting in its dephosphorylation and activation. A detailed and instructive analysis of these and some other results [80] has been published by Burg [1]. Unfortunately, the results of Colclasure and Parker have not been extended to other channels.

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What about the alternatives? Due to a change in the available volume, a step in osmolarity automatically results in an immediate change in the concentrations of all intracellular molecules and ions, including sodium and potassium. They can influence cellular processes in two ways. First, there is direct low-affinity binding of monovalent ions to proteins [81-84]. It is difficult to imagine, however, how such a mechanism could act as the first-response volume sensor because the relative magnitude of the concentration change can be small. It would require a highly specific allosteric amplification to convert a 10-20% change in the concentration of sodium or potassium into a rapid biological response. Besides, if this were the case, one would expect that an increase in permeability for sodium or potassium should produce a "recoil" in the form of an RVD or an RVI, respectively. But there is no evidence of that: potassium channel blockers increase cell volume, as one would expect [60], and potassium ionophore valinomycin produces a steady shrinkage [Rana and Model, unpublished].

The second mechanism may operate through the screening of local electrostatic fields between proteins. The quantitative measure of this is ionic strength, which takes into account the activities and valencies of all ions. Arguments have been made for [85-88] and against [89] the role of ionic strength in the regulation of swelling-activated anion channels. The question is made more complicated by the fact that intracellular ion activities are seldom known. The development of more specific probes [90] may help obtain better insight into the role of this factor.

An increase in membrane tension upon swelling can indeed activate some ion channels [91],but lateral compression of the membrane is hardly possible (unless shrinkage acts to reduce the preexisting tension, and the cell responds to this decrease relative to the resting state). Moreover, it has been observed that lowering the temperature, which should increase the membrane stiffness and tension, either slows down the regulatory volume changes or has no effect [92-95].

That leaves the cytoskeleton as the main non-MC candidate for initiating at least the RVI response. Generally, hypertonic shrinkage causes actin polymerization, while hypotonic swelling has the opposite effect [96-100]; there is ample (though not free from controversy) evidence for a role of the cytoskeleton in the RVI [101]. But that does not prove that cytoskeleton is activated by compression directly: cytoskeletal proteins, like any other proteins, are affected by MC. MC stimulates polymerization reactions in general, and *in vitro* experiments have shown that MC enhances not only polymerization of actin [102-105], but also polymerization of tubulin [106, 107] and of other cytoskeletal proteins [108, 109]; it also increases myosin ATPase activity [110] and promotes binding of glycolytic enzymes to the cytoskeleton [111]. If activation of ion channels depends on the cytoskeleton, the role of the latter may have to be relegated from a primary responder to an intermediary (Fig. 1).

But short-term assays tell only part of the story. They are most widely used on stationary cells because then CV becomes a convenient readout unobscured by cell growth and proliferation. Over a longer term, CV becomes unreliable, and the assay needs to be



Fig. 1. A diagram illustrating the hypothesis of how MC and actin may cooperate in regulating the RVD and RVI responses. F-actin is the polymeric form of actin.

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either conducted on large populations or in terms of MC. We have measured MC in HeLa cells incubated in a hyperosmotic medium between 20 min and 20 h. After an increase in MC lasting for at least 1 h (i.e., the apparent lack of RVI), it returned to the initial levels by 20 h [18] (our preliminary results also show the preservation of MC in HeLa cells kept overnight in a strongly hypotonic medium obtained by a 1:1 dilution with water). It is highly unlikely that either membrane or cytoskeletal forces can persist in growing and dividing cells for many hours, and MC seems best suited to guide the cells toward a stable condition. Cell types that do not display rapid RVD [112-114] or RVI [18, 52 115, 116] may provide a valuable tool for investigation of either MC maintenance (if CV eventually recovers) or the long-term consequences of altered MC.

Responses to gradual changes in osmolarity are different. They are less well studied than abrupt changes, although must be much more common in nature. The osmolarity of urine can vary from 0.05 M, when water is plentiful, to over 1 M, when it needs to be conserved, but that does not happen instantly. Likewise, the osmolarity of blood is continuously challenged by water loss and gain but is monitored and adjusted continuously through osmosensing signaling in the brain. Marine animals living in estuaries or fish migrating between rivers and the ocean are exposed to major but slow changes in water osmolarity.

The magnitude of volume changes is much smaller during slow transitions, for which reason this type of adaptation is called isovolumetric regulation [117, 118]. However, it is possible that, due to the inherent sensitivity of MC to small changes, fine-tuned feedback through MC helps maintain volume stability even during slow transitions. This question awaits further study.

Mechano- and osmosensing

Mechanical deformation of the membrane may result from a local force acting on a small area or from a whole-cell impact, such as resulting from osmotic swelling. Thus, some parallels exist between osmolarity sensing and mechanical sensing. Accordingly, membrane stretch and the cytoskeleton are the two most discussed mechanisms of mechanosensing, each supported by substantial experimental evidence [119]. At the same time, some results may allow interpretation in terms of MC. From the experiments on membrane blebs lacking cytoskeleton [120], the authors have concluded that cytoskeleton is irrelevant to the activation of the mechanosensitive channel PIEZO1 and, therefore, the tension in the lipid bilayer remains the only force capable of regulating this channel. However, cell blebs may contain a dramatically reduced protein concentration compared to the rest of the cell [121], and this fact brings MC back onto the scene. Furthermore, it has been shown that bacterial mechanosensitive channel MscS is directly regulated through MC-sensitive conformation of the cytoplasmic domain [122].

The high sensitivity of osmosensing in the brain is another fact that calls for an explanation. Osmosensitive neurons respond to volume perturbations as small as 1% (which would imply linear deformations on the order of 0.3%). Although a very minor stretch or compression may produce a significant force in a stiff material, cells would be expected to easily accommodate such small deformations. It has been hypothesized that a special arrangement of microtubules perpendicular to the membrane help convert mechanical forces to signaling by transient receptor potential (TRP) channels [73, 123]. Nevertheless, a recent investigation of the TRP channels failed to demonstrate their direct activation by stretch [124]. The conclusion made by the authors of the latter study was that TRP channels are unlikely to act as primary mechanotrasducers. This underscores the need to find one. It seems that the amplification by MC due to its nonlinear dependence on the volume, makes MC better suited for the role of a primary sensor, which can in turn transmit the stimulus to the cytoskeleton and cytoskeleton-tethered proteins.

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Hyperosmotically-induced apoptosis

Apoptosis is a form of programmed cell death that can be induced by a variety of chemicals and adverse conditions. It is characterized by the activation of specific cysteine proteases (caspases), condensation of chromatin, and cell shrinkage. At some point, apoptotic cells become dehydrated through a process known as apoptotic volume decrease [76, 125]. A 10-20% loss of volume due to water efflux appears to be quite common, and sometimes, the prevention of apoptotic volume decrease by potassium or chloride channel blockers may abolish all manifestations of apoptosis.

Not only does water loss occur during apoptosis development, but persistent osmotic shrinkage by itself can induce apoptosis [52]. We have recently studied hyperosmotically induced apoptosis in HeLa and MDCK cells [126] and came to the following conclusions. First, hyperosmotic compression produces apoptosis regardless of intracellular concentrations of sodium or potassium. Second, hyperosmotic apoptosis is not affected by actin depolymerization induced by cytochalasin. Although it is possible that cytoskeletal elements other than actin mediate signaling by shrinkage, that would seem less likely, especially for the lack of any evidence. Cytochalasin is commonly used to assess cytoskeletal involvement in many different processes and, moreover, hyperosmotic apoptosis has been observed in a variety of attached and suspended cells [52, 127-130], presumably with different patterns of cytoskeletal arrangements. We have not attempted to modify the membrane tension (which could be done by insertion of lipophilic compounds, such as anesthetics or ethanol [131]); however, as we have emphasized previously, the membrane could hardly act as a volumesensing mechanism in shrinkage. With ions, cytoskeleton, and membrane tension either eliminated or in doubt, an increase in MC comes out as the most probable mediator that converts hyperosmotic shrinkage to apoptosis.

Possible molecular targets of MC signaling in apoptosis

Any reaction that involves aggregation, oligomerization, or formation of stable macromolecular complexes can in principle be affected by MC, and these effects are more predictable than conformational changes or reaction rates. Aggregation/oligomerization reactions are essential in many processes, including transcription, translation, locomotion, and others [132], and it would be important to examine how they are influenced by MC. But a problem of this scope cannot be addressed in a single paper, and in this section we will focus on apoptosis as an example where shrinkage plays a particularly prominent role.

Depending on the stimulus, apoptosis can be initiated by one of the two pathways: extrinsic or intrinsic [133]. The extrinsic pathway is activated by ligand binding to the cell death receptors on the plasma membrane. By contrast, the intrinsic pathway is initiated within a cell upon cytotoxic or genotoxic stress, such as DNA damage, inhibition of a kinase, disruption of microtubules, or hyperosmolarity. Intrinsic apoptosis is regulated by a strict balance of pro- and anti-apoptotic proteins, known as the BH3-only proteins and the BCL-2 family proteins, respectively. In healthy cells, anti-apoptotic BCL-2 proteins, directly or indirectly, prevent the activation of the pro-apoptotic BAX family proteins (BAX and the related proteins, BAK and BOK), whereas, in cells undergoing apoptosis, one or more BH3only proteins are induced, which, in turn, activates BAX. Once activated, BAX is recruited to the mitochondrial outer membrane, where it oligomerizes and forms pores. The pores in the mitochondrial membrane initiate the mitochondrial outer membrane permeabilization, which triggers the release of cytochrome c from the mitochondrial intermembrane space. Once in the cytoplasm, cytochrome *c* binds to a monomeric adaptor protein Apaf-1. That causes Apaf-1 to shift from an auto-inhibitory to an active conformation and to form a wheel-shaped heptameric complex, the apoptosome. The apoptosome recruits the initiator caspase-9, followed by dimerization and activation of initiator caspase-9 molecules. Highly catalytic caspase-9 dimers start cleaving and activating executioner caspases, such as





Fig. 2. The main steps in the activation of intrinsic apoptosis (see text for detailed description). The red rectangles indicate the steps that involve binding and, therefore, are likely to be enhanced by hyperosmolarity: 1 – interaction between BH3-only proteins, BCL-2 family proteins, and BAX; 2 - insertion of BAX into the outer mitochondrial membrane; 3 – oligomerization of BAX within the membrane; 3 – binding of cytochrome c to monomeric Apaf-1; 4 – apoptosome formation; 5 – dimerization of caspase-9.

caspase-3, which proceed to dismantle cellular proteins, leading to apoptosis. It should be noted that the initiator caspases are activated by dimerization, whereas executioner caspases already exist as dimers and are activated by proteolytic cleavage by other caspases (Fig. 2).

How can MC affect the apoptotic cascade? If we look specifically at apoptotic reactions that involve the formation of protein complexes, the following reactions stand out:

- (1) Interaction between BH3-only proteins, BCL-2 family proteins, and BAX;
- (2) BAX translocation to the mitochondrial membrane;
- (3) Oligomerization of BAX within the mitochondrial membrane;
- (4) Apoptosome formation;
- (5) Dimerization of caspase-9.

Due to the nature of MC, any of these reactions may be enhanced by shrinkage that either develops during apoptosis (apoptotic volume decrease) or acts as a link between hyperosmolarity and activation of intrinsic apoptosis. However, we have shown that mitochondrial cytochrome c release triggered by hyperosmolarity can be completely inhibited by overexpression of anti-apoptotic BCL-2 [126]. Since cytochrome c release occurs upstream of Apaf-1 and caspase activation, the latter cannot be the sole mechanism of hyperosmolarity-induced induced apoptosis.

Conclusion

Despite decades of research and a solid theoretical foundation, as well as several convincing experiments, signaling by MC in volume regulation is often viewed as a second-tier hypothesis. Perhaps the biggest hurdle for its wider acceptance is the apparent lack

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of specificity of the effector, which runs against the desire of modern biology to reduce all cellular phenomena to a network of signaling and metabolic chains. In addition to that, there has been a dearth of studies of MC in situ; almost all that is known about MC has been obtained in test tubes using artificial macromolecules to mimic intracellular crowding. We have been trying to argue throughout this paper that regulation of CV by protein density is a viable hypothesis, and there are ways to study it in living cells, even if by the elimination of competing hypotheses.

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Disclosure Statement

The authors declare no conflict of interests exists.

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