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REVIEW

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The influence of cell volume changes on tumour cell proliferation

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Abstract Ion channels and cell volume control participate in a wide variety of cellular functions, including cell proliferation. According to the "pump-leak model" or the "double Donnan system", the cell volume is constant in physiological medium so long as the cell metabolism and the Na-K pump are not inhibited and the passive Na^+ permeability is not dramatically increased. At short term, this model has been supported by a large number of experiments made on different cell types. However, at long term, it may be insufficient to describe the volume control because it does not take into account the fact that cells possess a large number of membrane transporters and interconnected volume regulatory mechanisms. In this review, we present recent results indicating that, in physiological conditions, ion channels may have important roles in cell volume control. Furthermore, we emphasize that cell proliferation and volume are phenomenologically correlated. On the basis of the macromolecular crowding theory, the possibility that the cell osmolyte and water content mediates this correlation is discussed.

Keywords Cell division · Cell growth · Cell size · Ion channels

Abbreviations *4-AP* 4-aminopyridine · *NPPB* 5-nitro-2-(3-phenylpropylamino)benzoic acid · *TEA* tetraethylammonium · *TOR* target of rapamycin

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Introduction

Ion channels are extremely diverse and ubiquitous cell proteins. The fact that they are expressed in membranes of all cells indicates that they play a role in most, if not all, aspects of cell physiology (Dubois 1999). However, given that they have several interconnected biophysical functions, it is often difficult to determine the physicochemical link(s) between their activity or expression and cell functions or dysfunctions. In order to illustrate this problem, we describe here, in isotonic and anisotonic conditions, the role of ion channels in cell volume control and the involvement of this control in cell proliferation. On the basis of our recent results obtained on neuroblastoma and glioma cells (Rouzaire-Dubois and Dubois 1998; Rouzaire-Dubois et al. 2000, 2003), we discuss the possibility that the relationship between cell volume and rate of proliferation can be defined by Boltzmann-like equations, which may reflect the effects of macromolecular crowding on proteins controlling the cell cycle progression.

Biophysical functions of ion channels

Ion channels have at least three biophysical functions. They control the membrane potential, the intra- and extracellular ion concentrations and the cell volume (Fig. 1). Because of the ion selectivity of several channel types, passive transmembrane fluxes of ions create an accumulation of cations or anions in the vicinity of the plasma membrane and consequently create a transmembrane potential and transduce electrical signals. If ion channels are voltage dependent, this function is reciprocal, i.e. a change in membrane potential induced by a modification of channel activity influences the channel open probability. The second function of ion channels is to control the bulk intra- and extracellular ion concentrations. This is particularly true for calcium. Because its resting intracellular concentration is less than 100 nM, a

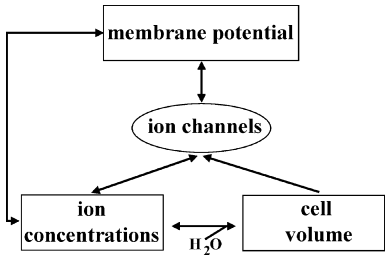

Fig. 1 Biophysical functions of ion channels. Ion channels have at least three interconnected functions: control of membrane potential, ion concentrations and cell volume

relatively small influx of Ca^{2+} through plasma membrane channels or Ca^{2+} release through endocellular channels significantly increases the cytoplasmic Ca^{2+} concentration, which activates Ca^{2+}-dependent channels. At medium or long term, sustained modifications of the open probability of channels selective to monovalent ions may also change intra- or extracellular monovalent ion concentrations. If changes in intracellular ion concentrations modify the cytoplasmic tonicity, osmotically obliged water fluxes through plasma membrane aquaporins, lipids and ion channels increase or decrease the cell volume, which is essentially determined by the cell water content. In most cell types, alterations of cell volume modulate the activity of volume-dependent Cl^- and K^+ channels. As a consequence, ion and osmotically obliged water fluxes regulate the cell volume. Finally, ion concentrations and membrane potential are interconnected via electrochemical gradients.

Cell volume control in isotonic conditions

It is now well established that animal cell homeostasis is governed by two principles: isosmolarity between intra- and extracellular solutions and electroneutrality in the bulk intra- and extracellular media (see Armstrong 2003). Since the cell membrane has a high water permeability, the first principle (isosmolarity) implies that any change in intracellular osmolyte content is associated with an osmotically obliged water flux and a change in cell volume. If one neglects the small excess of charges that makes the membrane voltage, the second principle (electroneutrality) implies that a change in cell cation content is associated with a similar change in cell anion content and vice versa. Taken together, these two principles imply that any change in net ion (and neutral osmolyte) fluxes is associated with a change in cell volume.

To survive, cells must concentrate inside themselves organic molecules (proteins, amino acids, sugars). The osmotic activity of these molecules, associated with the leak of Na^+ into the cells and the high water membrane permeability, creates a tendency to cell swelling. To reduce this tendency, animal cells use the Na-K pump to counteract the leak of Na^+. In this model, commonly referred to as the " pump-leak model" or the "double Donnan system" (see Armstrong 2003; Macknight and Leaf 1977), Na^+ ions, maintained extracellularly by the active transport out of cells, serve to counterbalance the intracellular colloid osmotic pressure. According to this model, the cell volume is assumed to be constant in a physiological external medium so long as the metabolism and the pumps are not inhibited and the influx of Na^+ through channels is not dramatically increased (Fig. 2). Concerning K^+ and Cl^- channels, it is assumed that they do not control the cell volume because it is supposed that Cl^- is in electrochemical equilibrium and any change in K^+ efflux through channels is compensated by a change in Na^+ influx. While it is recognized that the Cl^- equilibrium potential may be different than the resting potential (Armstrong 2003), the role of Cl^- channels in cell volume control is generally ignored. Moreover, it is assumed that, at rest, K^+ and Na^+ fluxes through channels are automatically compensated for by opposite fluxes mediated by the Na-K pump. If one considers the case where the activity of K^+ channels is decreased, the membrane should be depolarized, the Na^+ current through channels should be decreased and passive and active fluxes of K^+ and Na^+ should be reduced. Consequently, the cell K^+ and Na^+ contents should be unchanged and the cell volume should remain constant. However, if this paradigm has been largely confirmed at short term, it may be violated at mean or long term because it does not take into account the large number of transporters and the variety of processes that control the cell volume.

In contrast to the above paradigm, we showed that pharmacological blockade of K^+ and Cl^- channels in neuroblastoma and glioma cells induced a cell swelling, which developed within several hours (Fig. 3). If we consider that the blockers we used [4-aminopyridine (4-AP), Cs^+, 5-nitro-2-(3-phenylpropylamino)benzoic acid) (NPPB), tetraethylammonium (TEA)] (Rouzaire-Dubois and Dubois 1998; Rouzaire-Dubois et al. 2000,

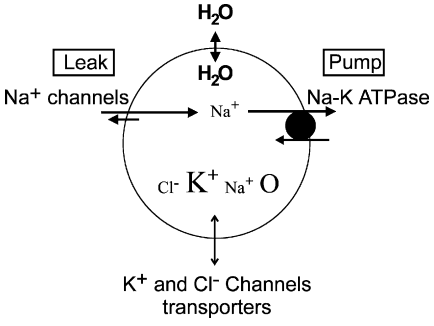

Fig. 2 Pump-leak model and cell volume control in isotonic conditions. The pump-leak model assumes that in physiological medium the cell volume is constant so long as the metabolism and the Na-K pump are not inhibited and the Na^+ influx through channels is not dramatically increased. If such alterations occur, the membrane is depolarized and the increase in NaCl and associated water content induce a cell swelling. It should be noted that this model does not take account of a possible role of K^+ and Cl^- channels and osmolyte transporters (*lower part of the scheme*) in cell volume control. *O* denotes organic osmolytes, i.e. charged and neutral amino acids and sugar

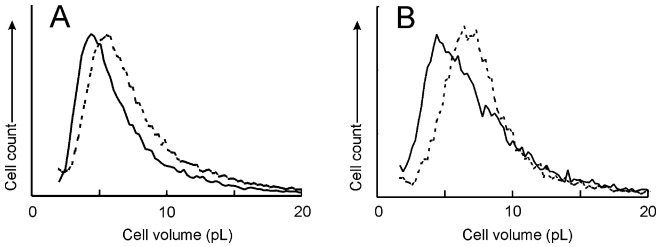

Fig. 3A, B Pharmacological blockade of K^+ and Cl^- channels induces cell swelling. NG108-15 neuroblastoma cell volume distributions under control conditions (*solid curves*) and after 24–48 h treatment with 10 mM TEA (**A**) or 50 μM NPPB (**B**) (*dashed curves*). The rightward shift of histograms in the presence of TEA and NPPB reflects an increase in mean cell volume of 15–20%. In this figure and the next one, cell volumes were determined with a Coulter counter

2003) had no other effects than to reduce K^+ and Cl^- currents, this implies that: (1) the Cl^- equilibrium potential is more positive that the resting potential and (2) the reduction of the K^+ current is not associated with a decrease in Na^+ current. Similar conclusions can be drawn from the results of Xu et al. (1996), who showed that 4-AP increased the volume of myeloblastic cells, and the results of Koegel et al. (2003), who showed that, in keratinocytes, the down regulation of Ca^{2+}-activated K^+ channels induced a cell size increase.

Altogether, these observations indicate that the cell volume homeostasis is more complicated than that predicted by the "pump-leak model" and, at least at mean or long term, the activity of various channels and transporters or other physico-chemical processes (Macknight and Leaf 1977; Wehner et al. 2003) should be taken into account to understand how cell volume is controlled.

Cell volume regulation in anisotonic conditions

Given that water is in thermodynamic equilibrium across the plasma membrane, any alteration in extracellular osmolarity results in fast transmembrane water flow and changes in cell volume, i.e. swelling in hyposmotic and shrinkage in hypertonic media, respectively. Then, most types of cells regulate their volume by processes of regulatory volume decrease (RVD) and regulatory volume increase (RVI). RVD is due to activation of Cl^- and K^+ channels and/or KCl co-transporters. RVI is largely mediated by activation of Na-K-Cl co-transporters and/or Na-H and Cl-HCO_3 exchangers. The respective intracellular ion loss and gain are associated with osmotically obliged water fluxes and volume regulation. In general, RVD and RVI occur over a period of a few to several tens of minutes, and then the cell volume is assumed to remain equal to its isotonic value while the extracellular medium is still anisotonic.

Since most cellular functions (in particular division) occur over a period of several hours, it was of interest to know whether, after the processes of RVD and RVI, the cell volume remains constant and if volume regulations are always the same in a given cell type. In neuroblastoma and glioma cells, we made several unexpected observations (Rouzaire-Dubois and Dubois 1998; Rouzaire-Dubois et al. 2000, 2003) (Fig. 4). First, the cell volume was not constant during continuous cell culture. It was maximal after 1 or 2 days following cell passage and then decreased (see also Conlon and Raff 2003). Second, when the cell volume was maximal, the cells up-regulated their volume in anisotonic conditions so that they swelled in hypertonic but shrank in hypotonic media, respectively. Third, after several days in culture, where the cell volume was reduced, the cells poorly regulated their volume in anisotonic conditions so that they remained shrunken and swollen for several hours in hypertonic and hypotonic media, respectively. All these observations confirm the above conclusion that cells possess a variety of regulatory volume processes mediated by a large number of membrane transporters and channels whose activity and expression are controlled by intracellular osmolarity, cell osmolyte and/or water content.

Cell size–proliferation relationship

For several decades, it has been known that there exists a close connection between cell size and division (Grewal and Edgar 2003; Hartwell and Unger 1977;

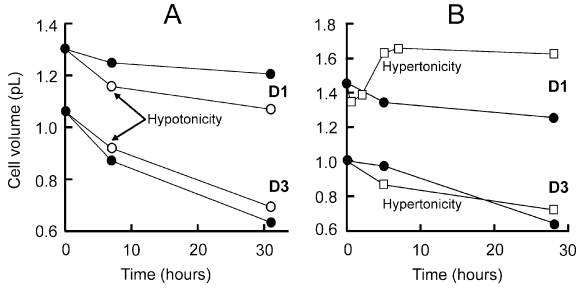

Fig. 4A, B Differential volume regulations of C6 glioma cells submitted to long-term anisotonicity during continuous cell culture. Hypotonicity (addition of 33% H_2O) (**A**, *open circles*) and hypertonicity (addition of 50 mM KCl) (**B**, *open squares*) were applied at time 0 after 1 day (*D1*) or 3 days (*D3*) of cell culture. Note that the mean cell volume was smaller at D3 than at D1. This cell shrinkage was assumed to be due to the production by the cells of inhibitory factors (Rouzaire-Dubois et al. 2003). In order to avoid dilution of these inhibitory factors, hypotonicity at D3 was made by substitution of the control medium with a conditioned hypotonic one from a 3-day culture of the cells. At D1, the cells up-regulated their volume so that, as compared to controls (*filled circles*), they shrank in hypotonic and they swelled in hypertonic medium. In contrast, at D3 the cells poorly regulated their volume and, compared to controls, they remained swollen and shrunken for several hours in hypotonic and hypertonic media, respectively. Mean ± SEM (the SEM bars are smaller than the points) of three experiments made in parallel in isotonic and anisotonic media. In **A**, the initial swelling that lasted a few minutes (Rouzaire-Dubois et al. 2000) is not shown

Neufeld and Edgar 1998; Nurse 1975; Polymenis and Schmidt 1999). In general, cells must double their size (or mass) before they divide so that they maintain a constant average size over generations. Moreover, we observed that neuroblastoma and glioma cell proliferation was inhibited when their size increased beyond an optimal value (Rouzaire-Dubois and Dubois 1998, Rouzaire-Dubois et al. 2003). In rat glioma cells, we obtained results in different experimental conditions, including: continuous cell culture, Cl^- channel blockade, anisotonicity and in the presence of an inhibitory conditioned medium collected from cell cultures or in a medium containing a low level of foetal calf serum, that define a bell-shaped relationship between cell proliferation and volume (Rouzaire-Dubois et al. 2003). This suggests that proliferation (P) is a function of at least three factors: a maximum rate of proliferation (P_{max}), an activation (A) and an inhibition (I) parameter, where A and I are dependent on cell size so that proliferation is optimal within a cell volume window. This new concept is comparable to the steady-state open probability of voltage-activated and -inactivated channels as a function of potential. Here, the modulator factor would be the cell volume and proliferation can be described by the following equations:

$$P = P_{max} AI \quad (1)$$

with:

$$A = 1/[1+(\exp(V_{A\,0.5} - V)/k_A)] \quad (2)$$

and:

$$I = 1/[1+(\exp(V - V_{I\,0.5})/k_I)] \quad (3)$$

In these equations, V is the cell volume, $V_{A\ 0.5}$ and $V_{I\ 0.5}$ are volumes corresponding to $P_{max}/2$ and k_A and k_I are slope factors.

Moreover, we showed that this model could describe any change in rate of proliferation, suggesting a phenomenological correlation between cell volume and division (Fig. 5). In contrast to the conclusion of Conlon and Raff (2003) and Grewal and Edgar (2003) that, in normal mammalian cells, cell-size checkpoints controlling division probably do not exist, our results favour the concept that glioma cells possess at least two (a low and a high) volumetric checkpoints. What is more, our results suggest that proliferation and cell cycle progression are not all-or-none mechanisms but are modulated by cell volume changes, reflecting the effects of intracellular osmolytes or water on proteins or genes involved in division. Since our results were obtained on clonal tumoural cells, it will be interesting to know if our model applies to normal proliferating cells and can be generalized to all cell types. If this is not the case, as suggested by the observations of Conlon and Raff (2003) on Schwann cells, our results could have potential implications in cancer-related research.

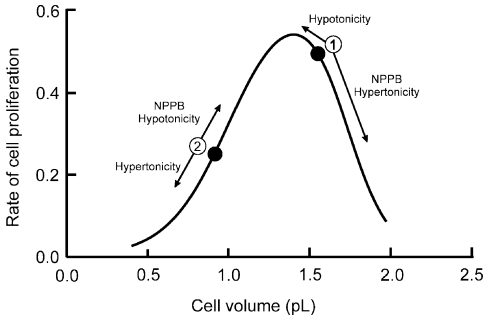

Fig. 5 Phenomenological correlation between cell size and proliferation. According to our recent results (Rouzaire-Dubois et al. 2003), the rate of glioma cell proliferation (P), calculated from the cell number (N) at day D and day $D+1$ and expressed as: $P=\log_{10}(N_{D+1}/N_D)$, changes with mean cell volume at day D in a bell-shaped manner and can be described by Eqs. (1)–(3) (*continuous curve*) with $P_{max}=0.655$, $V_{A\ 0.5}=1.0$ pL, $V_{I\ 0.5}=1.72$ pL, $k_A=0.19$ pL and $k_I=0.13$ pL. Cell size alterations induced by NPPB or anisotonicity differentially modified cell proliferation at D1 (*state 1*) and at D3 (*state 2*) of cell culture (*arrows*)

How may cell size control proliferation?

Assuming a bell-shaped and causal relationship between cell growth (increase in cell size) and division (increase in cell number), the remaining question is how cell volume can influence proliferation. Cell growth and division are controlled by two distinct pathways: the target of rapamycin (TOR) pathway and the mitogen-activated protein kinase (MAPK) and cyclin pathway. However, these pathways are not strictly independent since, in human osteosarcoma cells, inhibition of growth blocks division, and in rat fibroblasts, inhibition of division increases cell growth (Fingar et al. 2002). In contrast, in rapamycin-treated mice, transfection with cyclin D1 increased hepatocyte proliferation and size (Nelsen et al. 2003). At the membrane level, mitogens and growth factors stimulate the activity or expression of nutrient transporters, non-selective cations, K^+ and Cl^- channels (Edinger and Thompson 2002; Frace and Gargus 1989; Wilson and Chiu 1993; Wondergem et al. 2001). According to the principle of isosmolarity, the resulting fluxes of nutrients and ions are associated with water fluxes and cell volume changes. This can modify the intracellular concentration of solutes, such as enzyme co-factors involved in cell cycle progression. However, the effects of these solutes must be highly cooperative to take into account the effects of relatively small changes in cell volume on proliferation. One possibility to explain this cooperativity is to consider that the activity of cell cycle controlling proteins is dependent on their crowding and on the cell water content.

As emphasized above, the total concentration of macromolecules inside the cell is high. The intracellular medium is termed "crowded" rather than "concentrated" because the concentration of no single macromolecular species is high, but, taken together, the macromolecules occupy 20–30% of the total cell

volume, the remaining 70–80% being mainly occupy by water. Consequently, cell swelling leads to dilution and cell shrinkage to concentration of cellular constituents, including proteins. It is well known that conformation and activity of several macromolecules are dependent on their crowding and on the ratio of hydration versus osmotically active water. Because of these properties, relatively small changes in their concentration lead to large changes in their activity (Ellis 2001; Garner and Burg 1994; Minton 2001; Parsegian et al. 2000). Consequently, changes in cell volume should alter the activity of various macromolecules, including proteins in cell growth and division pathways. Let us now see whether the macromolecular crowding theory can predict a bell-shaped relationship between cell volume and proliferation and thus can justify Eqs. (2) and (3). Within the framework of this theory, at least three cases can be considered.

Assuming that a macromolecule can exist under two conformations: a hydrated and active (*a*) form and a dehydrated or partially dehydrated inactive (*b*) form, changes in cell water content (and thus cell volume) should alter the (*a*/*b*) ratio of their concentrations. Since this ratio develops as an exponential in the free energy difference (ΔG_{ab}) necessary for the molecule to go between the two forms (Parsegian et al. 2000), the probability to find the molecule in the active form is:

$$a/(a+b) = 1/[1+\exp(-\Delta G_{ab}/kT)] \quad (4)$$

where k is the Boltzmann constant and T the absolute temperature. It should be noted that Eq. (4) is similar to Eq. (2) if $-\Delta G_{ab}$ is proportional to the cell volume. If such a molecule stimulates the cell cycle progression, cell proliferation would be dependent on cell volume according to Eq. (2). In contrast, if another molecule, sensitive to crowding, inhibits the cell cycle progression, cell proliferation would be dependent on cell volume according to Eq. (3). According to these equations, the rate of proliferation is optimal within a volume window whose width is determined by the differential sensibilities to the cell volume of proteins that stimulate and inhibit cell cycle progression. Moreover, if cell cycle progression is modulated by the cell volume, it would be necessarily controlled by other factors and proliferation should be proportional to a maximal value (P_{max}) according to Eq. (1).

Another possibility is to consider that the cell cycle progression is dependent on the activity (γ_A) of a soluble protein (A) that forms a complex with a substrate (B) according to the equilibrium:

$$A + B \rightleftharpoons [K_D] \ AB \quad (5)$$

where K_D is the dissociation constant of the complex.

The effective concentration $[A]_{eff}$ of A is:

$$[A]_{eff} = \gamma_A [A]_{act} \quad (6)$$

where $[A]_{act}$ is the actual or total concentration of A. γ_A is a function of the equilibrium free energy ($< g_A >$) of non-specific interactions between A and all other macromolecules present in the medium according to the equation (Minton 2001):

$$\gamma_A = \exp(< g_A > /kT) \quad (7)$$

Equations (6) and (7) can be combined to yield Eq. (8):

$$[A]_{eff} = [A]_{act} \exp(< g_A > /kT) \quad (8)$$

According to the mass action law, the concentration of the complex AB is described by:

$$[AB] = [B]/\left(1 + K_D/[A]_{eff}\right) \quad (9)$$

where [B] is the total concentration (free + bound) of B. Combining Eqs. (8) and (9) yields Eq. (10):

$$[AB] = [B]/1 + K_D [A]_{act} (< -g_A > /kT) \quad (10)$$

If $< -g_A >$ is proportional to cell volume and the cell cycle progression is limited by the concentration of AB, proliferation would increase with cell volume as predicted by Eq. (2). As assumed in the preceding case, proliferation would be inhibited by cell swelling that increases the activity of an inhibitor of cell cycle progression.

A bell-shaped relationship between cell size and proliferation can also be predicted if the cell cycle progression is controlled by a reaction of the form (Ellis 2001):

$$A + B \ \rightleftharpoons \ AB* \ \rightleftharpoons \ AB \quad (11)$$

where AB* is the transition complex and AB stimulates the cell cycle progression. In such a case, cell swelling would increase the diffusion of A and/or B but decrease the thermodynamic activity of AB*. If the reaction rate is diffusion-limited at high degrees of crowding (small cell volume) and the transition-state is limited at low degrees of crowding (large cell volume), the cell size–proliferation relationship would be biphasic as predicted by Eqs. (2) and (3).

Given that crowding might influence the activity of several macromolecules involved in cell cycle progression, a cell volume increase should likely influence proliferation by several mechanisms, including the three simple models given above. However, since the cell cycle progression is controlled by many different parameters, more complex situations are likely to affect the bell-shaped relationship between cell volume and proliferation.

Conclusion and perspectives

For several decades, it has been known that ion channels control membrane potential and transduce electrical signals. More recently, it has been shown that ion channels may also control the cell volume in normal physiological conditions and that cell volume changes

modulate the rate of proliferation. In a recent paper (Rouzaire-Dubois et al. 2003), and in the present review, we have developed a model that accounts for a biphasic and phenomenological correlation between cell size and proliferation. This model is based on the macromolecular crowding theory and predicts that any change in cell volume, induced by alteration of ion and osmolyte channel and transporter activity, will influence cell proliferation to an optimal value within a volume window.

Cell growth (increase in cell size) and division (increase in cell number) are respectively controlled by two distinct but interconnected signalling pathways. We postulate that the cell water content and the cell volume mediate this interconnection. Consequently, it would be interesting to see whether and how proteins involved in these pathways are modulated by the activity of ion and osmolyte channels and transporters.

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