On the Role of G-Protein Coupled Receptors in Cell Volume Regulation

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Key Words

RVD • Thrombin • ATP and volume • Taurine

Abstract

Cell volume is determined genetically for each cell lineage, but it is not a static feature of the cell. Intracellular volume is continuously challenged by metabolic reactions, uptake of nutrients, intracellular displacement of molecules and organelles and generation of ionic gradients. Moreover, recent evidence raises the intriguing possibility that changes in cell volume act as signals for basic cell functions such as proliferation, migration, secretion and apoptosis. Cells adapt to volume increase by a complex, dynamic process resulting from the concerted action of volume sensing mechanisms and intricate signaling chains, directed to initiate the multiple adaptations demanded by a change in cell volume, among others adhesion reactions, membrane and cytoskeleton remodeling, and activation of the osmolyte pathways leading to reestablish the water balance between extracellular/intracellular or intracellular/intracellular compartments. In multicellular organisms, a continuous interaction with the

external milieu is fundamental for the dynamics of the cell. It is in this sense that the recent surge of interest about the influence on cell volume control by the most extended family of signaling elements, the G proteins, acquires particular importance. As here reviewed, a large variety of G-protein coupled receptors (GPCRs) are involved in this interplay with cell volume regulatory mechanisms, which amplifies and diversifies the volume-elicited signaling chains, providing a variety of routes towards the multiple effectors related to cell volume changes.

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Introduction

The ability to regulate volume is an ancient trait conserved throughout evolution in animal cells from essentially all species. This reflects the importance for cell homeostasis of maintaining a strict volume control, including that of intracellular compartments, to preserve the cytoarchitecture required for the correct assembly of signaling complexes. Although systemic osmolarity in physiological conditions is highly controlled, intracellular osmolarity is continuously compromised by the generation of local and transient osmotic microgradients derived from

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uptake of nutrients, secretion, synthesis and degradation of macromolecules, remodeling of the cytoskeleton and extracellular matrix and transsynaptic ionic gradients [1, 2]. Cell volume disturbances, particularly cell volume increase, occur in numerous pathologies. Hyponatremia associated with a large number of pathological situations characteristically results in cell swelling. Cell volume gain also occurs without a change in external osmolarity, as consequence of ion redistribution or increased cellular content of molecules acting as organic osmolytes. This isosmotic swelling, also known as cytotoxic swelling, occurs in hypoxia, ischemia, epilepsies, cranial trauma or hepatic encephalopathy. Cell volume changes have been considered for long time as a challenge for animal cells, compromising their homeostasis. Recent evidence, however, points to the intriguing concept of cell volume as a signaling element for basic cell functions such as migration, proliferation or apoptotic death.

Regulatory volume decrease

The high permeability to water characteristic of most animal cells requires the presence of mechanisms allowing the cell to face contingencies derived from water entry. Uncontrolled changes in cell water, by disturbing the intracellular concentration of signal molecules being at a certain point free in intracellular compartments, may send equivocal signals, disturbing the cell function and the intercellular communication. When water enters the cell as result of an external osmolarity reduction, a complex mechanism for volume recovery is activated. This basic mechanism has been preserved in essentially all species throughout evolution. This adaptive mechanism termed regulatory volume decrease (RVD) is accomplished by the extrusion of intracellular osmotically active solutes up to reach the water balance imposed by the new conditions. The osmolytes involved are the main intracellular ions K^+ and Cl^- and a number of small organic molecules, including amino acids, polycohols, and amines [2, 3]. RVD initiates by sensing the cell volume change, followed by activation of transduction signaling chains allowing the cell to organize the multiple and concerted responses necessary to face the volume gain challenge (Fig. 1). The mechanism(s) or molecules acting as volume sensors are not fully identified. Integrins, growth factor receptors, changes in macromolecular crowding, ionic strength or $[Ca^{2+}]_i$ and phospholipases activity, are all considered as related to volume sensing mechanisms [4]. Detection of the cell volume change is followed by the

activation of signaling networks, in which $[Ca^{2+}]_i$ rise and phosphorylation of protein tyrosine kinases and their targets, have a prominent role (Fig. 1). Several of these kinases activate by the volume increase but the specific role for each of them has yet to be ascribed to the variety of adaptive cell responses to swelling first, and to volume correction later. These include among others, construction and deconstruction of cell adhesions, reorganization of the cytoskeleton, temporal deformation and remodeling of the membrane. Also, swelling is detected by the cell as a stressful situation, and consequently alerts the mechanisms for survival. Part of the volume transduction chains are directed to activate the osmolyte efflux pathways ultimately leading to cell recovery (Fig. 1). The hierarchy, interplay and connection of the signaling molecules activated by cell volume changes are not yet fully established.

RVD activated by hyposmotic swelling has been extensively investigated but less is known about cell volume control in cytotoxic swelling. The extent of volume recovery may vary, depending primarily on the mechanisms responsible for swelling. When swelling is driven by redistribution of ions, the main mechanisms for volume recovery involving ion extrusion are not working. Organic osmolyte efflux pathways still operate but their contribution may not be sufficient to accomplish an efficient volume regulation.

Modulation by GPCRs of the regulatory volume decrease

Activation of GPCRs initiates a broad spectrum of intracellular signaling chains influencing a diversity of cellular responses. Elicited signals are determined by the receptor interaction with the various families of G proteins, which in turn, transduce signals through both α and $\beta\gamma$ subunits. Proteins from the G_q family mediate PLC-dependent generation of IP₃ and DAG, mobilization of Ca^{2+} from intracellular stores and PKC activation. $G_{12/13}$ family provide a pathway to Rho-ROK and the G_i proteins control the cyclic AMP pathway by inhibition of the adenylyl cyclase. The effects of GPCR agonists increasing osmolyte fluxes and RVD here discussed, suggest a prominent role of the G_q family, since in most cases are mediated by increases in $[Ca^{2+}]_i$, which add to that elicited by swelling. This $[Ca^{2+}]_i$ increase is one of the most consistent cell responses to hyposmotic swelling, although osmolyte fluxes and RVD are Ca^{2+} -independent in a number of cells [5]. An intriguing observation from

Fig. 1. Schematic model of the molecular mechanisms involved in the three main steps necessary for the cell volume recovery after hypotonic swelling: 1. volume sensing, 2. osmotransduction and 3. osmolyte efflux pathway activation. The scheme illustrates the elements involved in each one of the steps required for cell volume adjustment and their suggested interplay. In addition to the reactions leading to the corrective osmolyte fluxes, other signaling chains are evoked related to adaptive responses of the cell to the volume changes occurring during swelling first and during RVD. These include adhesion processes, cytoskeleton remodeling, stress-sensing mechanisms and cell survival and protecting signals. EGFR: Epidermal growth factor receptor; GPCR: G protein coupled receptors; TRP: Transient receptor potential channel; PI3K: phosphoinositide-3 kinase; src: src for sarcoma oncogene; PLC: phospholipase C; PKC: protein kinase C; Ras, Rho: members of the Ras family of small GTPases; FAK: focal adhesion kinase; ERK 1/2: extracellular signal-regulated kinases 1 and 2; CaM: calmodulin; CaMK II: calcium/calmodulin-dependent protein kinase II; TKR: tyrosine kinase receptor.

our laboratory was that even in these cells, $[Ca^{2+}]_i$ rise over levels induced by hypotonic swelling as that generated by ionomycin, results in a dramatic enhancement of taurine efflux and a faster and more efficient RVD [6]. Interestingly, the ionomycin-induced increase in taurine efflux was only evident in swollen cells and not in those with unchanged volume, in spite of the large increases in $[Ca^{2+}]_i$ elicited by the ionophore in the two conditions. In this sense, what $[Ca^{2+}]_i$ rise propitiates is a potentiation of the taurine efflux and not only the linear increase of a Ca^{2+} -dependent mechanism. Results on the effect of the ionophores on taurine fluxes and RVD raised the question of whether increasing $[Ca^{2+}]_i$ by physiological mechanisms such as the activation of GPCRs, is able to evoke the same response, i.e. increasing osmolyte fluxes and RVD rate. This review

documents the early studies on the topic and the surge of recent reports supporting a modulatory role of GPCRs on cell volume regulation.

GPCRs and organic osmolyte efflux

Organic osmolytes involved in RVD are a group of small organic molecules with heterogeneous molecular structure: amino acids (taurine, glutamate, glycine, GABA), polyalcohols (sorbitol, myo-inositol), amines (creatine, phosphoethanolamine, glycerophosphorylcholine) and N-acetyl-aspartate. Despite their difference in structure it has been suggested that they permeate through the same pathway. Taurine is often considered as representative of organic osmolytes and

Receptor	Agonist	Transduction mechanisms	Cell Type	Ref.
TAURINE				
Purinergic P2Y	ATP	PLC-Ca ²⁺ - CaM, CaMK II, TK	Swiss 3T3 fibroblasts	19
	ATP	Ca ²⁺	Human breast cancer cells	10
	ATP	NI	NIH3T3 fibroblasts	7
	ATP	Ca ²⁺	Human tracheal cell line	9,13
	ATP	Ca ²⁺ , CaM, nPKC	HeLa cells	14
H1 histamine	Histamine	Ca ²⁺ , CaM, nPKC	HeLa cells	14
PAR-1	Thrombin	NI	Myoblastic cell lines	16
	Thrombin	Ca ²⁺ _i , PKC	Astrocytoma cell line	17,18
	Thrombin	NI	HeLa cells	14
	Thrombin	PLC-Ca ²⁺ , src/EGFR	Swiss 3T3 fibroblasts	19
Bradykinin	Bradykinin	Ca ²⁺ , CaM, nPKC	HeLa cells	14
M ₃ muscarinic	Oxo-M	Ca ²⁺ _i , Ca ²⁺ _e , PLC, PKC	SH-SY5Y neuroblastoma	20,21
Lysophospholipid	LPA, SP1	Ca ²⁺ _i , PKC	SH-SY5Y neuroblastoma	21
Prostanoid	PGE1	cAMP, PKA	L-murine fibroblasts	27
β-adrenergic	NE	cAMP	Flounder erythrocytes	26
GLUTAMATE				
Purinergic P2Y	ATP	Ca ²⁺ _i , CaM, CaMKII, PKC	Cultured astrocytes	22,23
PAR-1	Thrombin	Ca ²⁺ _i , PLC	Cultured astrocytes	24
M3 muscarinic	Oxo-M	TK	SH-SY5Y neuroblastoma	20
MYO-INOSITOL				
M3 muscarinic	Oxo-M	Ca ²⁺ , PKC	SH-SY5Y neuroblastoma	25

Table 1. Potentiation by GPCR agonists of swelling-induced organic osmolyte fluxes. PLC: phospholipase C; CaM: calmodulin; CaMK II: calcium/calmodulin-dependent protein kinase II; TK: tyrosine kinase; NI: not identified; nPKC: novel protein kinase C; Ca²⁺_i: intracellular calcium; EGFR: epidermal growth factor; Oxo-M: oxotremorine-M; Ca²⁺_e: extracellular calcium; LPA: lysophosphatidic acid; SP1: sphingosine 1-phosphate; PGE1: prostaglandin E1; PKA: protein kinase A.

has particular features which are very convenient to trace its role as an osmolyte. Taurine is present in high concentrations in most animal cells, largely free in the cytosol. It is not a protein constituent and is essentially inert in terms of metabolism. Taurine is the most sensitive among organic molecules, to volume perturbations, with the lowest release threshold and the largest amount released [7, 8]. Swelling-induced taurine efflux occurs through a leak pathway, presumably an anion channel-like pore. This pathway is known as volume-sensitive organic anion channel (VSOAC).

Swelling-evoked taurine release is partly Ca²⁺ dependent in some cell types but in many of them is largely Ca²⁺-independent. In all cases, however, increasing [Ca²⁺]_i over levels raised by hyposmolarity by treatment with Ca²⁺ ionophores, markedly potentiates the swelling-induced taurine efflux. Interestingly, there is recent evidence showing a similar potentiation when [Ca²⁺]_i is raised by agonists of GPCRs in a variety of cell types. As shown in Table 1, the volume-sensitive taurine efflux

is increased by metabotropic purinergic receptors, likely of the P2Y2 or P2Y4 subtype, in airway epithelial cells [9], human breast cancer cells [10] 3T3 fibroblasts [11], hepatoma HTC cells [12], tracheal cells [13], HeLa cells [14] and hippocampal neurons [15]. Taurine efflux is also increased by PAR-1 activation in myoblasts [16], HeLa cells [14], astrocytoma [17], and neuroblastoma SH-SY5Y cells [18] and in 3T3 fibroblasts [19], and by M3 muscarinic, H1 histamine and lysophospholipid receptors [14, 20, 21]. Glutamate (D-aspartate) efflux is potentiated by agonists of purinergic and PAR-1 receptors in cultured astrocytes [22-24] and by the muscarinic cholinergic receptor agonist Oxo-M in SH-SY5Y cells [20]. This condition also increases the swelling sensitive efflux of myo-inositol from SH-SY5Y cells [25].

These effects of GPCR agonists increasing organic osmolyte fluxes appear to be essentially mediated by the PLC-Ca²⁺ pathway, being all of them Ca²⁺-dependent to some extent. The degree of dependence, though, varies according to the agonists examined, the osmolyte traced

Receptor	Agonist	Cell Type	Method	Transduction mechanisms	Ref.
Purinergic	ATP	Cultured astrocytes	PC-WC	NI	74
PAR-1	Thrombin	CPAE cells	PC-WC	Ca ²⁺ -independent	34
	Thrombin	Intestine 407 cells	¹²⁵ I efflux	NI	33
	Thrombin	SH-SY5Y cells	¹²⁵ I efflux	Ca ²⁺ -PKC independent	18
	Thrombin	Ehrlich-Lettre cells	PC-WC	NI	35
	Thrombin	Swiss3T3 cells	PC-WC	PLC-Ca ²⁺ , src/EGFR	19
Endothelin	ET1, ET2	Atrial cells	PC-WC	MEK-dependent	36
		Intestine 407 cells	¹²⁵ I efflux	NI	33
Bradykinin		Intestine 407 cells	¹²⁵ I efflux	Ca ²⁺ -dependent, TK	33
M3 muscarinic	Oxo-M	SH-SY5Y cells	¹²⁵ I efflux	Ca ²⁺ _i -PKC	18

Table 2. Potentiation by GPCR agonists of $\text{ICl}^-_{\text{swell}}$. PC-WC: patch-clamp, whole cell; NI: not identified; PAR-1: protease-activated receptor 1; MEK: MAPK/ERK-kinase; PLC: phospholipase C; PKC: protein kinase C; EGFR: epidermal growth factor receptor; ET: endothelin; M3: muscarinic cholinergic; TK: tyrosine kinase; Oxo-M: oxotremorine M.

and the magnitude of osmolarity reduction. In 20-30% hyposmotic solutions, the effect of purinergic or M3 muscarinic receptor activation increasing and glutamate efflux is largely Ca²⁺-dependent (86-90%), regardless of the cell type [20, 23]. A PLC-dependent, thapsigargin-sensitive increase in $[\text{Ca}^{2+}]_i$, accounts for most of the ATP effects increasing taurine efflux. CaM and CaMKII are also part of this signaling cascade [11]. The increased efflux of taurine by thrombin-induced PAR-1 activation is 50% Ca²⁺-dependent in 3T3 fibroblasts [19] and more dependent (80%) in astrocytes (unpublished). A role for PKC on the stimulatory effect of GPCR activation on taurine and glutamate efflux is suggested by its attenuation by PKC blockers or down-regulation of the enzyme. This is observed for the thrombin, LPA and Oxo-M-stimulated taurine efflux in astrocytoma and neuroblastoma cells [17, 20, 21] and for the ATP increased D-aspartate efflux in cultured astrocytes [23]. The fact that PKC and $[\text{Ca}^{2+}]_i$ depletion are additive in some systems suggests the operation of two different pathways and the participation of Ca²⁺-independent PKC isoforms [23]. Taurine efflux increased by SP1 or LPA is less dependent on the Ca²⁺ pathway, being only 34-38% reduced when $[\text{Ca}^{2+}]_i$ elevation is prevented by treatment with BAPTA-AM or thapsigargin [21]. This feature may reflect the unique ability of these agonists to concurrently stimulate G_i, G_q and G_{12/13} proteins. A more complex signaling mechanism for the PAR-1 potentiation of taurine efflux is found in 3T3 fibroblasts. An EGFR/src signaling pathway accounts for about 50% of the taurine efflux evoked by thrombin [19]. The two signaling chains, i.e. the Ca²⁺-dependent and the EGFR/src-mediated pathways operate independently. This is not observed in astrocytes in which similar to that found for the P2Y potentiation depends primarily on Ca²⁺-mediated pathways.

The modulatory influence of GPCRs on organic osmolyte efflux may not be restricted to the Ca²⁺-signaling mediated receptors. In flounder erythrocytes, norepinephrine markedly increases the swelling-activated taurine efflux via a cAMP-mediated pathway [26]. A recent study in murine fibroblasts shows that receptors acting via the G_i family, activating cyclic AMP-dependent mechanisms also potentiate the volume-sensitive taurine efflux, with a magnitude similar to that of the PLC-Ca²⁺-signaling pathway [27] (Table 1).

It is important to remark that the increase in organic osmolyte efflux elicited by GPCR agonists occurs essentially through the VSOAC, being abolished by blockers of this pathway. It is also worthy to mention that all studies here reviewed have been performed in cells swollen by hyposmolarity. It is still unknown whether the effects of GPCR activation are reproduced in conditions of isosmotic swelling.

GPCRs and $\text{Cl}^-_{\text{swell}}$

The swelling-activated Cl⁻ channels ($\text{Cl}^-_{\text{swell}}$) are present in a large variety of cells and in most of them exhibit similar properties. The $\text{ICl}^-_{\text{swell}}$ current is outwardly rectifying, of intermediate conductance, with inactivation kinetics and a requirement for activation of free intracellular ATP. $\text{Cl}^-_{\text{swell}}$ have a broad permeability to anions, including large anions as benzoate or glutamate, and are sensitive to general Cl⁻ channel blockers [28].

The effect of intracellular perfusion of GTPγ activating a Cl⁻ current with the features of $\text{ICl}^-_{\text{swell}}$ first suggested a role for G proteins on the volume-sensitive Cl⁻ efflux pathway [29-32]. This was supported by a study in Intestine 407 cells showing that a variety of GPCR

Fig. 2. Thrombin increases the amplitude and reduces the time for activation of the hypotonicity-induced Cl^- current in Swiss 3T3 fibroblasts. **A.** Current density vs. voltage curves from cells in isosmotic medium (□), isosmotic medium + thrombin (■), 30% hypotonic medium (H30) (○) and 30% hypotonic + thrombin (HT) (●). **B.** Time course of $\text{ICl}^-_{\text{swell}}$ activation by hypotonicity or by hypotonicity + thrombin. Traces show the current density at 100 mV in the indicated experimental conditions. **C.** Time to half activation in 30 % hypotonic medium (clear bar) and 30 % hypotonic medium + thrombin (dark bar). Results are means $\pm$ SE of 6-8 experiments. Experimental procedure details in [17].

agonists, ATP, thrombin, bradykinin and endothelin, all increase the swelling-induced efflux of ^{125}I [33]. It was later shown that thrombin increases the hypotonic ^{125}I efflux from neuroblastoma cells [18] and that thrombin and endothelin increase $\text{ICl}^-_{\text{swell}}$ amplitude in pulmonary artery endothelial, Ehrlich-Lette ascites and atrial cells [34-36]. Noradrenaline exhibit a complex effect on $\text{ICl}^-_{\text{swell}}$ in portal vein myocytes, inducing an increase in $\text{ICl}^-_{\text{swell}}$ amplitude by acting on α -1 adrenoceptors and a decrease by stimulating β -adrenoceptors [37] (Table 2).

A detailed description of the effect of thrombin in $\text{ICl}^-_{\text{swell}}$ in 3T3 fibroblasts is shown in Fig. 2. This Cl^- current exhibits the features characteristic of $\text{ICl}^-_{\text{swell}}$. It is an outwardly rectifying current, with inactivation kinetics at large positive voltages and a reversal potential of -4 mV, close to the predicted Cl^- equilibrium potential in the condition of the experiment. Thrombin influenced $\text{ICl}^-_{\text{swell}}$

in fibroblasts by markedly enhancing the current density, and decreasing the time to half activation. Thrombin effect is exerted apparently on the same level of current density, as would be expected if it is not affecting the number of channels in the membrane. As shown in Fig. 2, thrombin alone did not induce by itself large currents, as it has been previously reported in endothelial cells [34].

In contrast to the primarily Ca^{2+} -dependent effect of GPCR activation on the organic osmolyte fluxes, the effect on Cl^- efflux pathways is described as Ca^{2+} -dependent or Ca^{2+} -independent. The hypotonic ^{125}I potentiation by bradykinin in Intestine 407 cells depends completely on $[\text{Ca}^{2+}]_i$ rise and is PKC independent [33]. In contrast, ^{125}I efflux increased by thrombin from SH-SY5Y neuroblastoma cells is basically independent of Ca^{2+} and PKC [18]. In pulmonary artery endothelial cells the thrombin-induced increase of $\text{ICl}^-_{\text{swell}}$ is unaffected

when the thrombin-induced $[Ca^{2+}]_i$ rise is prevented and cells maintain a basal $[Ca^{2+}]_i$ of 50-100 nM [34] but in 3T3 fibroblasts, the effects of thrombin are reduced in essentially Ca^{2+} -free conditions [19]. The effect of endothelin in atrial cells is insensitive to PKC down-regulation [36]. These differences in the mechanism of the modulatory effect of GPCR agonists might be attributed either to the cell type, to differences in the cell swelling degree, or to the magnitude of Ca^{2+} buffering.

A common feature of the GPCR agonists potentiation of ICl^-_{swell} is that no effect is observed in isosmotic conditions. It is only when swelling has activated the efflux pathway that the modulatory effect can be observed. However, in cultured corneal keratocytes, a chloride current identified as ICl^-_{swell} is activated by LPA and SP1 in isosmotic conditions [38]. Since as previously mentioned, these agonists may interact with other G-protein families and their effects on RVD-related events have to be examined in more detail.

GPCRs and volume-activated K^+ channels

Understanding the influence of GPCRs on volume corrective K^+ fluxes is complicated by the fact that events concurrent with cell swelling, such as membrane depolarization, $[Ca^{2+}]_i$ rise or membrane stretching, activate a variety of K^+ channels which are potential contributors to K^+ efflux from swollen cells. It is actually documented that voltage-dependent, stretch-activated, Ca^{2+} -activated K^+ channels of small, intermediate or large conductance and 2P-4TM channels, contribute to the K^+ efflux elicited by swelling in different cell types [39, 40]. Particularly the Ca^{2+} -activated K^+ channels of large conductance (BK) are primarily involved in the swelling-evoked K^+ efflux and RVD [39, 40]. Most of these channels are modulated by GPCR ligands, but the consequence of this effect on the volume-sensitive K^+ efflux and cell volume regulation has been examined in only few cases. BK channels are stimulated by endothelin, bradykinin, histamine, and isoproterenol in a variety of cells, but this effect is analyzed regarding mainly their action on vasoconstriction, or on other cell responses and not in connection with RVD. In any event, it is expected that when BK channels are responsible for the volume-sensitive K^+ efflux, GPCR activation in swollen cells will certainly influence volume regulation. Thrombin and ionomycin stimulate K_v channels in human platelets, but similarly, no connection is established with volume regulation [41].

Studies directly related to GPCR agonist effects on K^+ channels activated by volume changes are so far limited. In cultured astrocytes, the volume-sensitive ^{86}Rb efflux is Ca^{2+} -independent, but is markedly potentiated by the ionomycin-induced $[Ca^{2+}]_i$ increase [42]. A K^+ channel directly activated by swelling has been described in Ehrlich ascites cells, which is insensitive to the classical K^+ channel blockers but is inhibited by clofilium [43]. It is proposed that this channel is tonically inhibited by a pertussis-sensitive G protein [43]. Activation of purinergic P2Y receptors potentiates BK channels or ^{86}Rb efflux in a kidney cell line (Vero cells), in Ehrlich ascites [44, 45], and Intestine 407 cells [33] and in cultured human cancer cells [46]. Bradykinin, thrombin or histamine activate a charybdotoxin-sensitive ^{86}Rb efflux in Ehrlich ascites cells [45, 47]. These results altogether, show that, in contrast to the universality of the volume-sensitive Cl^- and organic osmolyte efflux pathways, the variety of K^+ channel types involved in volume regulation difficults the analysis of the potential effect of GPCR activation. An additional factor further complicating the interpretation of the effect of GPCR agonists is that, in contrast to that found for organic osmolytes or Cl^-_{swell} , the GPCR ligands activate K^+ channels in non swollen cells.

GPCRs and RVD

An early study by Bender and coworkers in cultured astrocytes [48] demonstrated, to our knowledge for the first time, the involvement of GPCRs in RVD. This study showed that endothelin, thrombin, bradykinin, ATP and carbachol, all accelerate RVD via a PLC-dependent mechanism. Further support to a G-protein role in RVD came from a study in cervical cancer cells showing facilitation of RVD by GTP γ S [49]. Subsequent studies showed that ATP facilitates RVD in airway epithelial and in Intestine 407 cells [50, 51]. Thrombin has this same effect accelerating RVD in 3T3 fibroblasts [19]. RVD facilitation is also promoted by the $[Ca^{2+}]_i$ rise elicited by ionomycin or A23187 [6], suggesting that Ca^{2+} -dependent events mediate the effect of GPCR agonists. This is confirmed by the effect reducing RVD of inhibitors of the PLC- Ca^{2+} signaling pathway [19], an expected result in view of the key role of $[Ca^{2+}]_i$ rise on the effect of GPCR activation increasing osmolyte fluxes, as above discussed. A more complex signaling mediates the effect of thrombin accelerating RVD in 3T3 fibroblasts, which besides the PLC- Ca^{2+} -CaM-CaMKII pathway, involves src and EGFR as a parallel signaling route [19].

This will be further discussed below.

The monomeric G-proteins of the Rho family

The low-molecular weight G-proteins of the Rho subfamily control multiple cellular processes, many of them related to their action as regulators of the actin cytoskeleton organization. Changes in cell volume during swelling and the subsequent RVD, involve dramatic changes in the cytoskeleton, which are necessary for the cell to adapt successfully to the dynamics of these events [52]. Rho (isoforms A,B,C) and their targets, as well as isoforms from the Cdc42 and Rac subfamilies participate in this process, interacting in a complex dynamic balance [53-55]. Either through this effect or independently of cytoskeleton changes, Rho-signaling participates on $\text{Cl}^-_{\text{swell}}$ activation in various cell types, as shown by the effects of bacterial toxins which specifically block Rho GTPases, or by the influence of a constitutively active Rho mutant expression [54-56]. Evidence at present suggests the requirement of a functional Rho pathway for $\text{Cl}^-_{\text{swell}}$ operation but not for its activation, postulating a permissive role for this pathway. On this respect, an active Rho pathway may facilitate $\text{Cl}^-_{\text{swell}}$ operation by promoting the formation of an actin-based platform for the proper assembly of elements involved in $\text{Cl}^-_{\text{swell}}$ trigger.

The involvement of Rho GTPases in K^+ and taurine efflux pathways is supported by a study in NIH3T3 cells expressing constitutively active Rho. A marked increase in the hyposmolarity-evoked taurine and ^{86}Rb efflux is observed in these cells [56]. Not only the efflux rate constant was enhanced but the osmolarity threshold was reduced as well. Osmolyte fluxes in isosmotic conditions are comparable in the wild type and the Rho activated cells, being in this respect, similar to the effect of receptors linked to heterotrimeric G-proteins. In congruence with the effect of active Rho increasing two important osmolyte efflux pathways, volume regulation after swelling was markedly increased in cells expressing the active Rho pathway [56]. Consistent with the notion of a permissive role for the Rho signaling pathway, the hyposmotic stimulus does not directly activate the Rho pathway [54] but a number of molecules responding to swelling are known to activate them including integrins, growth factor receptors and guanine nucleotide exchange factors. In contrast to the heteromeric G-proteins, monomeric G-proteins of the Rho subfamily are not directly activated through ligand binding, though a subset of GPCRs including those activated by lysophosphatidic acid and

thrombin participate in cytoskeleton remodeling and other cell responses associated with volume changes, via Rho-dependent pathways.

Is the permissive pathway mediated by tyrosine kinases?

A remarkable and consistent feature of the effect of GPCR agonists on organic osmolyte fluxes and $\text{ICl}^-_{\text{swell}}$ is that it is exerted only when the efflux pathway is already in operation. The precise nature of this permissive pathway is not yet fully established, but there is evidence of a prominent role for tyrosine kinases (TK). The involvement of TK in volume regulation activated after hyposmotic swelling has been recognized since the early report by Tilly *et al.* [57] showing inhibition by general TK blockers of the osmosensitive fluxes of K^+ and Cl^- in an epithelial cell line. Subsequent studies confirmed these results for specific TK and for organic osmolytes in a variety of cell types [58]. TK such as FAK, and members of the Src family, activate in response to hyposmotic swelling [58-61]. Another important family of signaling molecules, the growth factor receptors, which are transmembrane molecules with intrinsic TK activity, are also responsive to this stimulus. EGFR phosphorylation is increased by hyposmotic cell swelling in fibroblasts and keratinocytes [61, 62] and phosphorylation of ErbB4 increases by swelling in cerebellar granule neurons [63]. The phosphorylation state of these TK modulates osmolyte efflux pathways and RVD, which are inhibited by TK blockers, and correspondingly potentiated by blockade of tyrosine phosphatases [58]. The TK signaling chain can be linked to volume sensing molecules, such as integrins or the growth factor receptors themselves. A sequence of integrin - FAK - src activation by swelling is well established [59, 60]. A src-EGFR connection as swelling signal is also documented [19]. The extensive signaling network formed by these kinases may be part of the permissive pathway on which GPCR agonists exert their influence.

Tyrosine kinases and GPCR cross-talk

The involvement of tyrosine kinases in osmolyte efflux pathways and volume regulation is relevant to the topic of this review considering the evidence now available about a cross talk between GPCRs and TK, a mechanism known as transactivation. EGFR and members of the Src

Fig. 3. Hypotonic swelling evokes ATP release by exocytosis, or a conductive, anion channel-like pathway modulated possibly by CFTR or ATP-binding cassette members. Released ATP attains levels sufficient to activate purinergic receptors of the P2Y subfamily. The signaling chain evoked by these receptors has an influence on the hypotonic $[Ca^{2+}]_i$ rise, on osmolyte fluxes and RVD. There is still controversy of whether the sole activation of these receptors by external ATP is able to elicit ICl^-_{swell} or organic osmolyte fluxes in non swollen cells.

family are the molecules more directly involved in this interplay [64-67]. GPCR/tyrosine kinase crosstalk is only starting to be examined in reference to cell volume regulation. A recent study from our group in 3T3 fibroblasts [19] shows that activation of PAR-1 by thrombin potentiates the hypotonic phosphorylation of EGFR and src. The contribution of this transactivation pathway to volume regulation is evaluated by the effect attenuating thrombin effects on taurine efflux, ICl^-_{swell} and RVD by the blockers AG1478 (EGFR) or PP2 (src). This transactivation pathway may be linked to thrombin activated PLC- Ca^{2+} -mediated signaling chain, or may independently influence the volume regulatory mechanisms. In the study in fibroblasts, the two pathways operate separately but this may not be the case for other cell types. The proposed GPCR/tyrosine kinase interaction is an avenue for diversifying the signaling cascades activated by cell volume changes. This diversification may be most convenient considering that cell volume is now known to be involved in cell functions as diverse as

proliferation, migration and apoptosis, which may require different signaling routes.

GPCR modulation of RVD in physiology and pathology

Autocrine GPCR agonists

The stimulatory effect of GPCR agonists on osmolyte fluxes and RVD is at present firmly established. A question is then raised as whether these receptors are part of the volume recovery signaling chain or act essentially as modulators, adding new signals to the chain activated after swelling. Purinergic and leukotriene receptors have been proposed to operate as receptors for autocrine factors released by swelling. Derived from eicosanoids, the cysteinil leukotrienes in particular LTD₄, are released to the extracellular space, bind to a leukotriene receptor and via trimeric G proteins initiate a signaling pathway resulting in modulation of the taurine efflux or

Cell type	ATP _e	ATP scavenger, receptor blockers*	Ref
ICl _{swell}			
HTC rat hepatoma	-	Inhibition	38
Tracheal epithelial cells	-	Kinetics modulation	13
Biliary epithelial cells	-	Inhibition	77
Intestine 407 cells	Potentialiation	Inhibition	79
Human hepatocytes	-	Prevent activation	71
Cultured astrocytes	Activation	Inhibition	74
Hippocampal neurons	Activation	Inhibition	15
Taurine and glutamate efflux			
Glutamate			
Cultured astrocytes	Potentialiation	No effect	22
Taurine			
Swiss 3T3 fibroblasts	Potentialiation	Inhibition	11
Tracheal epithelial cells	Potentialiation	-	9
Tracheal epithelial cells	Potentialiation	Inhibition	13
Hippocampal neurons	Potentialiation	Inhibition	15
Human breast cell line	Potentialiation	Inhibition	10
RVD			
HTC rat hepatoma	-	Inhibition	38
Biliary epithelial cells	-	Inhibition	77
Intestine 407 cells	Facilitation	Inhibition	51
Human hepatocytes	-	Inhibition	71
Turbot hepatocytes	-	Inhibition	76
Airway epithelial cells	Facilitation	Inhibition	50

Table 3. Influence of ATP and P2Y receptors on volume regulation events *Apyrase, suramine, reactive Blue and pyridoxal phosphate.

activation of a K⁺ current. This proposed signaling cascade is supported by the effect reducing taurine efflux and RVD of lipoxygenase blockers or by inhibition of the synthesis of arachidonic acid and leukotrienes [7, 36, 68].

An autocrine/paracrine role for ATP on volume regulation is proposed based on the numerous reports on a swelling-induced efflux of ATP [50, 69-74] (Fig. 3). Since the early study by Wang *et al.* [69] the swelling-induced ATP release has been consistently observed in various cell types including carcinoma and hepatoma cells, airway epithelial cells and cultured astrocytes [69-74]. Quantification of the amount of ATP released has shown that the levels attained in the immediate vicinity of the cell surface are sufficient to activate P2Y purinergic receptors [50, 74]. The volume-sensitive ATP release may occur via exocytosis or more commonly through a conductive, anion channel-like pathway, possibly regulated by CFTR or ATP-binding cassette proteins [69, 70, 73] (Fig. 3). While there is general agreement about the effect of swelling inducing ATP release, the key question of whether P2Y receptor activation is a trigger or a modulator of osmolyte fluxes and RVD is still

controversial. In favor to the autocrine role is the effect reducing osmolyte fluxes and volume recovery of agents or conditions impairing the purinergic-mediated transmission shown in a variety of cell types [50, 69-76] (Table 3). Against this notion is the fact that external ATP in some cases [22, 23, 77, 78] but not always [74], is *per se* unable to raise osmolyte fluxes in non swollen cells. In this respect, however, the consideration raised about other GPCR agonists on the requirement of a prior activation of the permissive pathway are valid for ATP. Furthermore, in support to the autocrine role for ATP is the fact that, in contrast to other GPCR agonists, swelling is the trigger for its release. The volume-sensitive ATP release is of particular interest in brain due to the critical role played by ATP in astrocyte signaling and in the astrocyte-neuron interaction. ATP participates importantly in glia-glia communication via Ca²⁺-wave propagation, which is mediated by ATP release in various preparations [79]. Widespread propagation of Ca²⁺ waves through astrocytes is important in pathological states such as seizure and brain trauma, two conditions associated with cell swelling.

Potentially harmful effects of GPCR agonists

The GPCR interaction with signaling molecules activated during RVD diversifies and amplifies its signaling network and makes the process faster and more efficient. In certain situations, however, the potentiation of osmolyte fluxes induced by GPCR agonists represents a risk for potential harm. The marked potentiation by thrombin of glutamate efflux from astrocytes is an example of such situation [24]. Besides acting as an osmolyte, glutamate is a major excitatory neurotransmitter, and if released in excess to the extracellular space induces overfunction of excitatory neuronal receptors resulting in cell death. In pathological situations such as head trauma, hemorrhagic episodes and ischemia, there is a marked increase of brain thrombin levels, which together with the astrocyte swelling characteristic of these conditions, precipitates the excessive glutamate efflux and excitotoxicity. Hyperexcitability may also occur by a thrombin-induced enhancement of glutamate release from swollen nerve endings. In addition, the widespread propagation of Ca^{2+} waves through astrocytes evoked by ATP and possibly by other GPCR agonists as well, increases in pathological states such as seizure and brain trauma [79], two conditions concurrent with cell swelling.

Volume-related cell functions potentially modulated by GPCRs

Synaptic transmission

Swelling and GPCR activation may importantly modulate the synaptic function. In isolated nerve endings, hyposmotic swelling increases the efflux of amino acids such as GABA and glutamate, which serve the double role of osmolytes and neurotransmitters [80]. Moreover, swelling evokes a series of events such as depolarization and Ca^{2+} rise, via exocytosis, which also increase the synaptic efflux of molecules like norepinephrine which are essentially neurotransmitters and do not participate in volume regulation [81]. Among the GPCR ligands known to increase osmolyte efflux are those which activate neurotransmitter metabotropic receptors, such as cholinergic and noradrenergic receptors. Thus, swelling-induced neurotransmitter release and its potentiation by metabotropic receptors may have a potent modulatory role of synaptic transmission, in both physiological and pathological conditions.

Cell proliferation, migration and apoptosis

Cell migration involves numerous reactions closely interconnected with changes in cell volume. Extensive remodeling of the cytoskeleton, construction and deconstruction of cell adhesions are indispensable for a coordinated cell migration. Furthermore, changes associated to cell migration generate intracellular local osmotic gradients creating volume changes in the microenvironment, which demand an immediate adjustment to guarantee the polarity and direction for the proper displacement of the migrating cell [82, 83].

Volume control has been related to cell proliferation. Cell volume is determined genetically and is an attribute for each cell type. Before dividing, cells must attain the characteristic volume of the parent cells. A phenomenological correlation between cell volume and proliferation is suggested by results showing that swelling elicited by either hyposmolarity or blockade of volume-sensitive K^{+} channels decrease proliferation [84]. Diverse types of K^{+} channels, including a swelling-activated channel, have been implicated in tumour cell proliferation [85] and $\text{Cl}^{-}_{\text{swell}}$ channels have been also considered to participate in the cell cycle progression based on evidence showing that $\text{Cl}^{-}_{\text{swell}}$ are activated during cell proliferation, and conversely, $\text{Cl}^{-}_{\text{swell}}$ blockers interfere with proliferation [86-89]. Therefore, GPCR ligands known to participate in cell proliferation in a variety of cell types, such as thrombin, angiotensin, bradykinin, lysophosphatidic acid and sphingosine 1 phosphate may interact with volume-sensitive pathways. Another site of GPCR influence is the stimulation of growth factor receptors, a reaction known as transactivation. Hyposmotic swelling in some cells activate EGFR and this effect is potentiated by GPCR agonists [11, 19]. This concerted action may result in a more effective activation of the various EGFR downstream signaling pathways eventually influencing cell survival and proliferation. The interplay between GPCRs and volume-activated mechanisms, so far largely unexplored, warrants future investigation.

Cell volume decrease has been always recognized as a hallmark of apoptosis, but it is only recently that this volume reduction, termed apoptotic volume decrease, is considered as part of the apoptotic signaling. Apoptotic volume decrease is an early event in the program, occurring before other characteristic traits such as caspase 3 cleavage, cytochrome c release and translocation, endonuclease activation and DNA

fragmentation [90], Apoptotic volume decrease relies on the same mechanisms of RVD, i.e. the active translocation of intracellular osmolytes, K^+ , Cl^- and taurine [90-95], which noteworthy, activate in non swollen or even shrunk cells. Modulation by GPCRs on these mechanisms has not been examined in detail, but represent a most intriguing venue for future research.

Conclusion and perspectives

Cell volume is determined genetically for each cell lineage, but it not a static feature of the cell. It is of extreme importance that volume is kept unchanged to maintain cell homeostasis, but when changes occur, they affect basic aspects of the biology of the cell and may represent a risk for the whole organism. The recent surge of interest about a modulation of the basic mechanisms for cell

volume control by the most important and extended family of signaling elements, the G proteins, opens a plethora of possibilities for diversifying the signaling routes for the variety of cell functions involving volume changes. Synaptic transmission, intercellular communication, proliferation, migration, secretion and apoptosis, all may be influenced by this diverse and intricate interplay between GPCRs and the mechanisms for cell volume control. This is an intriguing avenue for future research.

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