Review
Integration of signalling pathways regulated by small GTPases and calcium

Pontus Aspenström*

Biomedical Center, Ludwig Institute for Cancer Research, Box 595, S-751 24 Uppsala, Sweden
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Abstract

The Ras superfamily of small GTPases constitutes a large group of structurally and functionally related proteins. They function as signalling switches in numerous signalling cascades in the cell. During the recent years, an increased awareness of a communication between signalling systems employing Ras-like GTPases and signalling systems employing calcium has emerged. For instance, the intensity of the activation of Ras-like GTPases is regulated by calcium-dependent mechanisms, acting on proteins that facilitate the activation or inactivation of the small GTPases. Other Ras-like GTPases have a direct influence on calcium signalling by regulating the activity of certain calcium channels. In addition, several small GTPases collaborate with calcium signalling in regulating cellular processes, such as cell adhesion, cell migration and exocytosis.

Keywords: Ras; Rho; GTPase; Calcium; Actin

1. Introduction

The Ras gene products constitute the founding fathers of the small GTPases, a large group of structurally and functionally related proteins [1–3]. The triad of proto-oncogenes, H-Ras, K-Ras and N-Ras, was identified over 20 years ago [4]. Activated mutants of Ras, in particular of K-Ras, have been frequently identified in a variety of human carcinomas [4]. The number of proteins found to be related to Ras has grown considerably over the last two decades and have now condensed into six subfamilies: Ras, Rab, ARF, Ran, Rho and Rad/Gem/Kir (RGK) [3]. The enzymatic properties of most of the different members of the Ras superfamily are regulated by the same fundamental principle: they are active when bound to GTP and hydrolysis of GTP to GDP results in a conformational change causing the inactivation of the proteins [2]. The hydrolysis activity of most members of the small GTPases is rather low, but it is increased severalfold by the aid of GTPase activating proteins, or GAPs. Conversely, the replacement of the hydrolysed GDP for a GTP is aided by guanine exchange factors, or GEFs. Thus, the GAPs function as negative regulators and the GEFs function as positive regulators of Ras signalling and together they will aid the small GTPases to alternate between inactive and active conformations [3]. However, the simple cycling between GTP- and GDP-bound stages is not the only determinant in signalling by small GTPases: the spatial and temporal distributions of the different small GTPases, as well as of their regulators, are equally important determinants.

The knowledge regarding the Ras superfamily has grown considerably over the years. Collectively, the members of the Ras superfamily function as signalling switches in many vital cellular processes. The Ras subfamily has mainly been implicated in regulating cell proliferation and the cell cycle [2]. Calcium (Ca^{2+}) has also been shown to be a critical factor for regulating cell proliferation [5,6]. The first hints of a signalling relationship between Ras-like GTPases and Ca^{2+} signalling came...
from observations that transformation caused by oncogenic Ras interfered with Ca^{2+}-triggered terminal differentiation of keratinocytes [7]. Later on, a direct link was suggested by observations in phaeochromocytoma PC12 neuronal cells, which demonstrated that Ca^{2+} signalling directly resulted in the activation of Ras [8]. Work during the last 10 years has resulted in a more detailed picture of the interrelationship between pathways regulated by Ca^{2+} and Ras-like GTPases [9]. The Ca^{2+}-induced activation of Ras can, at least partly, be explained by the existence of Ca^{2+}-regulated RasGEFs, RasGAPs as well as Ras effector proteins, which aid in the integration of Ca^{2+} and Ras-regulated pathways. Interestingly, members of the RGK subfamily, such as Gem, have been shown to have a direct influence on Ca^{2+} signalling by regulating the expression and function of voltage-dependent Ca^{2+} channels [10]. In addition, the Rho GTPases, as well as some of their regulating proteins, have been shown to have roles in Ca^{2+}-dependent cell adhesion, cell migration and exocytosis [11,12]. This review is not intended to cover all the detailed aspects of signalling networking between small GTPases and Ca^{2+}; instead this review will highlight critical aspects of the collaborations between the small GTPases and Ca^{2+}.

2. Ras GTPases and calcium

There exist at least two possible ways to activate Ras in a Ca^{2+}-dependent manner (Fig. 1). One pathway involves the non-receptor proline-rich tyrosine kinase 2 (Pyk2) [13]. This protein is regulated by a variety of Ca^{2+} elevating stimuli and it binds to many signalling molecules, most importantly, to members of the Src non-receptor tyrosine kinase family. The Pyk2-Src complex, in turn, binds to a protein complex involving the adaptor protein Grb2 and the Ras exchange factor Sos, and can thereby confer Ras activation [13]. Another pathway results in Ras activation in a more direct fashion by involving RasGEFs, RasGAPs, as well as Ras-binding proteins, so-called effectors, which relay Ca^{2+}-dependent signals [9,14] (Fig. 1).

2.1. Calcium-regulated RasGEFs

The first clues regarding the mechanisms underlying the link between Ca^{2+} and Ras came from the identification of Ras-GRF (also known as CDC25Mm) and Ras-GRF2 [15–17]. Ras-GRF is highly expressed in neurons and contains calmodulin (CaM) binding motifs, so-called IQ motifs. It was shown that the Ras-GRF-mediated...
activation of Ras was markedly enhanced in response to Ca$^{2+}$ [15]. Ras-GRF is actually functioning both as a RasGEF and a RacGEF; however, it is currently not clear if the protein can mediate also Rac activation in a Ca$^{2+}$-dependent manner [17]. The GRP/CalDAG-GEF family represents an important family of Ras activators that might be regulated by Ca$^{2+}$ [9,14]. Some of the members can also confer activation of other members of the Ras subfamily, such as Rap and TC21 [14]. However, although the members of the GRP/CalDAG-GEF family have Ca$^{2+}$-binding EF hands and DAG-binding C1 motifs, there are conflicting data regarding the absolute requirement of Ca$^{2+}$ in regulating their function [9,14]. Another protein with a RasGEF domain, as well as a domain with phospholipase activity, was recently identified [18–20]. This protein, known as PLCe, also contains Ras-binding motifs, so-called Ras association (RA) motifs, suggesting that PLCe also can function as a Ras effector, i.e., downstream of Ras. PLCe has been proposed to coordinate signalling pathways that employ Ras, Rap and DAG/IP$_3$/Ca$^{2+}$ [18–20]. This is certainly an attractive possibility, however, it is something that remains to be studied in more detail.

2.2. Calcium-regulated GAPs

Calcium has not only been shown to stimulate Ras activation but, furthermore, there are several reports that Ca$^{2+}$ signaling participates in the reciprocal process: the inactivation of Ras. The ubiquitous p120RasGAP was linked to Ca$^{2+}$ signals by virtue of its C2 domain, a domain-type known to confer Ca$^{2+}$-dependent phospholipid-binding, and some reports have suggest an involvement of p120RasGAP in Ca$^{2+}$-dependent processes [9,21]. Recently, two RasGAP-domain containing proteins, CAPRI (Ca$^{2+}$ promoted Ras inactivator) and RASAL, have been shown to be under the dynamic influence of Ca$^{2+}$ signals [22,23]. CAPRI was shown to translocate to the plasma membrane in a Ca$^{2+}$ signalling-dependent manner and the membrane association was necessary for the GAP activity of the protein [22]. RASAL was also shown to translocate to the plasma membrane upon agonist-induced increase in the intracellular Ca$^{2+}$ concentration [23]. RASAL was shown to function as a Ca$^{2+}$ sensor, reacting to the repetitive increases in intracellular Ca$^{2+}$ triggered by agonists (so-called Ca$^{2+}$ transients), which resulted in oscillations of RASAL between cytosolic, inactive, and plasma membrane-associated, active stages [23]. In T-cells, activation of the T-cell receptor was shown to lead to a specific activation of Ras in the Golgi apparatus [24]. Interestingly, this organelle-specific activation of Ras was shown to be caused by the concerted action of RasGRP1 and CAPRI. RasGRP1, which was translocated to the Golgi by a Src and PLCe1-dependent mechanism, ensured the activation of the pool of Ras present in the Golgi, whereas the action of CAPRI, which inactivated the pool of Ras translocated to the plasma membrane, ensured a Ras activation confined uniquely to the Golgi [24].

3. Calmodulin-dependent regulation of small GTPases

Another level of Ca$^{2+}$ regulation of Ras-like small GTPases has been suggested by the finding that CaM can bind to, and inhibit the activity of, certain Ras isoforms. Calmodulin was found to bind specifically to the K-Ras4B isoform (but not to K-Ras4A, H-Ras or N-Ras) [25]. This binding specificity was further demonstrated by the finding that CaM inhibition resulted in the unique activation of K-Ras [25]. In addition, PKC was shown to be able to participate in Ras activation if CaM was simultaneously inhibited [26]. Thus, the CaM binding is likely to be a determinant for the isoform-specific activation of Ras. CaM was also found to bind the Ras-like GTPases RalA and RalB in a Ca$^{2+}$-dependent manner and CaM was proposed to have a role during thrombin activation of Ral in platelets [27]. Moreover, another Ras-like GTPase, Rin, was found to bind CaM. This interaction was required for the Rin-induced neurite outgrowth in neuronal, PC12 cells [28]. Finally, functional interactions between calmodulin and members of the Rad/Gem/Kir (RGK) subfamily of GTPases have also been identified [29,30].

4. Regulation of calcium homeostasis by small GTPases

An intriguing link between the activity of small GTPases and Ca$^{2+}$ homeostasis has been identified by studies of Gem (also know as Kir) [31]. This GTPase, together with Rad and Rem, are collectively referred to as the RGK GTPases [3]. Gem was found to bind to the $\beta$-subunit of the voltage-gated Ca$^{2+}$ channel [10]. This Ca$^{2+}$ channel is a multi-subunit protein complex, which regulates the influx of extracellular Ca$^{2+}$ into the cells [31]. The voltage-gated Ca$^{2+}$ channels are regulated at different levels: one level of regulation is achieved by increasing the ER retention time of the $\alpha$1-subunit, which results in a decreased expression of the protein product. The ER retention is decreased by binding of the $\beta$-subunit to the $\alpha$1 subunit, which masks the motif in the $\alpha$1-subunit responsible for ER retention. As a result, the expression is increased. Interestingly, Gem was found to interfere with this intricate regulation, since the binding of Gem to the $\beta$-subunit interfered with its potency to bind to the $\alpha$1-subunit. Hence, the $\alpha$1-subunit is retained in the ER, resulting in decreased expression of the Ca$^{2+}$ channel [10,31] (Fig. 1). The Gem-like GTPases Rem and Rad have also been found to bind to the $\beta$-subunit and to have roles similar to Gem in regulating voltage-gated Ca$^{2+}$ channels [32]. Importantly, the activity of the Gem protein can, in turn, be modulated by CaM, since binding of CaM
to the C-terminal of Gem will interfere with the GTP binding of Gem and, hence, the activity of the protein [29,30].

Interestingly, the ability to regulate the function of Ca\(^{2+}\) channels might not be restricted to Gem. Recently, it was found that the Ras effector RASSF1 has the capacity to bind the β subunit of plasma membrane Ca\(^{2+}\) pump 4b [33]. Thus, Ras signalling, via RASSF1, might regulate the Ca\(^{2+}\) influx. Moreover, R-Ras has been shown to have an influence over the Ca\(^{2+}\) release from internal stores. In a study by Koopman et al. [34], it was shown that cholecystokinin-induced Ca\(^{2+}\) release from ER was increased in an R-Ras-dependent manner.

5. Rho GTPases and calcium

Calcium not only regulates cell proliferation, it is also an important determinant of processes that regulate cell morphology and cell migration [6]. Ca\(^{2+}\) is an important regulator of cell–substratum adhesion as well as cell–cell adhesion. Intriguingly, the Rho GTPases also regulate these processes and pathways involving these proteins often coincide with Ca\(^{2+}\)-regulated pathways (Fig. 2). Sometimes these pathways run in parallel, working synergistically to regulate cell movements and cell adhesion, but there is an increasing awareness that they might be linked [12].

5.1. Regulation of cell adhesion, cell migration and actin organisation

Cell adhesion is to a large extent a Ca\(^{2+}\)-regulated process: adhesion to neighbouring cells is achieved by a group of plasma membrane-associated proteins collectively known as cadherins [35]. Most cells in vertebrates have the capacity to attach to other cells at least at some occasions; therefore, a correct cell–cell adhesion is of critical importance for the homeostasis of many cell types, in particular epithelial cells. Dysfunctional cell adhesion is often, if not always, associated with disease conditions, something which has been thoroughly studied over the years [35]. Cadherins are Ca\(^{2+}\)-binding proteins and the Ca\(^{2+}\)-binding is critical for their function. This notion implies that alterations in the extracellular Ca\(^{2+}\) concentration have a direct impact on the adhesive behaviour of epithelial cells. Cadherin-containing adhesive structures, so-called adherence junctions, are dynamic and are under a constant reconstruction. In addition, cadherins also influence several intracellular signalling cascades [35].

![Fig. 2. Rho GTPases and calcium. Cell adhesion leads to activation of the Rho GTPases. The Cdc42 and Rac effector IQGAP1 is under the influence of CaM and has the ability to affect cell adhesion and cell migration. Calpain has the potency to influence cell migration both in a positive and a negative fashion and the action is, at least to a part, relayed by the Rho GTPases. The Rho GTPases have an influence over Ca\(^{2+}\)-dependent exocytosis. The Miro GTPases have a role in regulation of mitochondrial homeostasis, a process which might be under the influence of Ca\(^{2+}\).](image-url)
The Rho GTPases have been found to be activated in a specific manner by cadherin signalling, thus alterations in extracellular Ca\(^{2+}\) will regulate cadherin function, which in turn will affect the activity of the Rho GTPases [36] (Fig. 2). The IQGAP family of proteins has been suggested to integrate signalling involving Rho GTPases, cytoskeletal regulation and Ca\(^{2+}\). IQGAP1, which is the best characterised representative of this three member group of proteins, binds Cdc42 and Rac1 and participates in the stabilisation of the GTP-bound conformation of the Rho GTPases [37–39]. IQGAP1 also binds to CaM, actin filaments, β-catenin and the mitrotubule-binding protein CLIP170 [37,40–42]. IQGAP1 has been shown to promote cross-linking of actin filaments, at least in vitro, and, importantly, IQGAP1 has been shown to decrease cadherin-mediated cell–cell adhesion, since IQGAP1 dissociates the interaction between α-catenin and β-catenin/cadherin [41,43,44]. Cdc42/Rac-binding has been shown to influence the interaction between β-catenin and IQGAP1 in a negative manner [45]. These observations have been built into a model stating that an increase in the intracellular Ca\(^{2+}\) concentration will result in increased CaM binding to IQGAP1, which, in turn, will result in a decreased Cdc42/Rac binding to IQGAP1 [11,46]. The released IQGAP1 will then weaken the cadherin-regulated cell adhesion. This model, however attractive, might be a bit oversimplified; it is for instance not clear if Ca\(^{2+}\)-sensitisation of smooth muscle cells and thereby contraction will affect the activity of the Rho GTPases [36] (Fig. 2). A first indication in this direction came from work on mast cells and showed that RhoGDI, which sequesters the Rho GTPases in inactive complexes, inhibited secretion [60,61]. Subsequently, it was found that Rac1 and Cdc42, but not RhoA, had supportive roles during exocytosis [61,62], and conversely, inactivation of Rac1 and Cdc42 with Clostridium toxins resulted in an abolishment of the Ca\(^{2+}\)-induced exocytosis [63,64].

5.2. Exocytosis

Exocytosis in secretory cells, such as mast cells, chromaffin cells from adrenal medulla and neuronal cells, has for a long time been known to be a process strictly regulated by Ca\(^{2+}\). During the recent years, the picture has emerged that critical aspects of this process are also regulated by small GTPases of the Rho family [58,59] (Fig. 2). A first indication in this direction came from work on mast cells and showed that Rac and Cdc42 were involved in Ca\(^{2+}\)-dependent exocytosis [63,64]. Moreover, work on mast cells and showed that RhoGDI, which sequesters the Rho GTPases in inactive complexes, inhibited secretion [60,61].

5.3. Additional links between Rho GTPases and Ca\(^{2+}\)

There are several additional possible links between Rho GTPases and Ca\(^{2+}\)-regulated and Ca\(^{2+}\)-regulating pathways, although this matter has not yet been explored in great detail. Several RhoGEF and RhoGAP domain-containing proteins have domains that confer direct binding to Ca\(^{2+}\), such as EF hands, or domains, such as C1, C2 and IQ motifs, which confer binding to signalling components in the Ca\(^{2+}\)-dependent pathways [see for instance http://smart.embl-heidelberg.de]. In line with these implications, the catalytic activity of a Cdc42- and Rac-
specific RhoGAP domain-containing protein, designated RICS (RhoGAP involved in the β-catenin-N-cadherin and NMDA receptor signalling), was recently shown to be under the control of Ca\(^{2+}\)/calmodulin-dependent protein kinase II [72]. Another RhoGAP-domain containing protein, nadrin, was shown to have a role in Ca\(^{2+}\)-dependent exocytosis in neuronal cells [73]. Collectively, these observations highlight the notion of collaborations between Rho proteins and Ca\(^{2+}\). Moreover, one family of Rho GTPases, Miro, have EF hands in addition to the GTP-binding domains and Saccharomyces cerevisiae Miro (also known as YAL048c) has been shown to confer growth in a Ca\(^{2+}\)-dependent manner [74,75].

6. Conclusions

Recent research on small GTPases has begun to acknowledge the importance of the spatial and temporal distribution of these signalling molecules to lend signal transduction pathways specificity. In addition, we start to understand more of the networking between different types of signal transduction pathways, such as pathways employing small GTPases and calcium. As a result, there is an emerging awareness that it is the concerted and balanced action of many signalling molecules that together make up the signalling pattern that gives the specific signalling flavour.

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