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Targeting of calcium:calmodulin signals to the cytoskeleton by IQGAP1

Maria I. Psatha [a], Minoo Razi [b], Anna Koffer [c], Steve E. Moss [d], David B. Sacks [a], Stephen R. Bolsover [c,*]

[a] *Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, United States*
[b] *Cancer Research UK, Lincoln's Inn Fields, London, United Kingdom*
[c] *Department of Physiology, University College London, Gower Street, London WC1E 6BT, United Kingdom*
[d] *Institute of Ophthalmology, University College London, United Kingdom*

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Abstract

Mast cells reorganize their actin cytoskeleton in response to cytosolic calcium signals while in parallel secreting histamine and other inflammatory mediators. The effect of calcium on actin is mediated in large part through calmodulin. EGFP-tagged calmodulin is concentrated in the actin-rich cortex of RBL-2H3 mast cells. Transfection with small interfering RNA directed against the actin and calmodulin-binding protein IQGAP1 dramatically reduced expression of the latter protein and reduced or eliminated the concentration of calmodulin at the actin-rich cortex. Both actin reorganization and secretion were enhanced in IQGAP1 knockdown cells. Our results suggest a model in which calmodulin is targeted to and sequestered at the actin cytoskeleton by IQGAP1. Upon cell stimulation and the subsequent $[Ca^{2+}]_i$ increase, it is immediately available to activate local downstream targets.

Keywords: Calmodulin; IQGAP1; Mast cell; RBL-2H3; Actin; Calcium

1. Introduction

Mast cells are secretory cells of the immune lineage. Activation with an Fcε receptor crosslinker or by co-application of phorbol ester and a calcium ionophore initiates two distinct responses. First, stimulation evokes a reorganization of the initially cortical actin cytoskeleton that results in cell spreading and, in the RBL-2H3 mast cell line, active ruffling. Second, stimulated cells secrete histamine and other inflammatory mediators [1–4]. Both disassembly of the cortical actin cytoskeleton and secretion can be elicited in permeabilized cells by an increase in calcium concentration alone. Calcium-induced disassembly of the cortical F-actin is mediated by calmodulin. Blocking calmodulin's action with excess calmodulin-binding peptide, or with the calmodulin antagonist W7, completely prevents calcium-evoked disassembly of the cortical actin cytoskeleton in permeabilized mast cells [5]. The main target of calmodulin appears to be myosin light chain kinase, since inhibition of this enzyme with the specific inhibitor ML-7 completely prevents calcium-evoked cortical actin disassembly [5]. Myosin light chain kinase does not bind calcium-free calmodulin and is inactive in the uncomplexed state. However, it has an unusually high affinity for calcium:calmodulin [6]. The calcium:calmodulin:kinase complex phosphorylates the regulatory light chain of myosin II, activating the motor protein. The force thus generated triggers contraction of the actin cortex followed by its disassembly and reorganization. Inhibition of myosin II with butane dione monoxime very significantly inhibits calcium-evoked cortical actin disassembly [5]. In contrast the main route by which calcium together with dia-

* Corresponding author. Tel.: +44 20 7679 6564; fax: +44 20 7383 7005.
E-mail address: s.bolsover@ucl.ac.uk (S.R. Bolsover).

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cylglycerol (both generated after activation of PLCγ and subsequent hydrolysis of PIP_2) evoke secretion is by activating protein kinases C, which then phosphorylate components of the exocytotic machinery [7,8]. Part of this action is direct, by the action of calcium and diacylglycerol on conventional protein kinases C [9]. Another part is indirect and operates through calmodulin, which activates calmodulin-dependent protein kinases which in turn phosphorylate and activate phospholipase D [7]. Phospholipase D generates phosphatidic acid and hence diacylglycerol that activates both conventional and novel protein kinases C [9]. Calcium may also act directly on some of the components of the SNARE complex that mediates the final steps of exocytosis [10]. These relatively direct effects of calcium are modulated by more complex mechanisms including actions of the Rho family of small GTPases. For example, calcium-induced dissociation of the cortical F-actin is slightly enhanced by active Rho through a ROCK-mediated phosphorylation of myosin light chain [11], while Rac1 and Cdc42 promote ruffling and spreading, respectively [12]. Calcium-evoked secretion is inhibited when Rho is inactivated by C3 transferase [11,13] while Rac1 and Cdc42 also stimulate calcium-evoked secretion [11,13].

In a previous study we examined the distribution of a calmodulin-EGFP chimaera within RBL-2H3 cells [14]. In agreement with studies on several other cell types we observed a central concentration at the centrosome/mitotic spindle pole [15–20]. However, we also observed a prominent concentration of calmodulin in the cell cortex [14]. This localization appeared to be due to an association of calmodulin with F-actin, since it could be eliminated by disruption of the actin cytoskeleton with latrunculin. In this study we identify IQGAP1 as the linker protein responsible for targeting calmodulin to the actin-rich cortex. IQGAP1 is the major calmodulin-binding protein in calcium-free breast epithelial cell lysates [21]. It contains a calponin homology domain (CHD) which binds actin [22]; four calmodulin-binding IQ motifs, a GRD (GAP related domain) homologous to the GTPase activating domains of Ras family GTPases; a RasGAP_C (distal C terminal) domain that is reported to bind E-cadherin, β-catenin and the microtubule-associated protein CLIP170 [23,24]. Although the IQ, GRD and RasGAP_C domains are distinct sections of polypeptide, binding of calmodulin is in fact competitive with Cdc42, E-cadherin, actin and β-catenin [25,26]. Calcium:calmodulin can also bind to the CHD domain if the latter is expressed as a separate fragment [25]. In the complete protein, however, it appears to be only the IQ domains that are important in calmodulin binding [27,28]. IQGAP1 appears to have a number of roles in the control of the cytoskeleton, including acting as an effector of Cdc42 and Rac1, members of the Rho family of small GTPases [29], and promoting bundling of actin filaments [27,30]. In this paper we report that knockdown of IQGAP1 expression not only prevents localization of calmodulin at the cell cortex, but also promotes both reorganization of the actin cytoskeleton and secretion.

2. Materials and methods

2.1. Cell culture

Generation of $^{CaM\text{-}EGFP}$RBL-2H3, a non-clonal RBL-2H3 cell line expressing a functional chimaera of calmodulin and enhanced green fluorescent protein, has been described previously [14]. Native and CaM-EGFP expressing RBL-2H3 cells were maintained in a growth medium of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum, 50 units/ml penicillin, 50 μM streptomycin and 4 mM glutamine. K2 rat fibrosarcoma cells [31] were maintained in MEM with Hanks' salts supplemented with 10% bovine serum (SML, Germany), 0.09% sodium bicarbonate (Sigma) and 1 mM glutamine.

2.2. RNA preparation

IQGAP1 was specifically knocked down by small interfering RNA (siRNA). Two IQGAP1 gene specific double stranded 21-nucleotide siRNAs were predicted (HiPerformance design algorithm, QIAGEN), confirmed by BLAST search to be unique to rat IQGAP1, and synthesized. These were RIQ873 = 5′-AATGCCATGCTTGTCAATCTT-3′ and RIQ3985 = 5′-GTGGATGAGTATTCCGATCTA-3′, targeted against coding regions 873–893 and 3985–4005 bp (relative to the first nucleotide of the start codon) of rat IQGAP1 (accession no. 34857387). The control 21-nucleotide oligonucleotide, 5′-CUGCUCUCUUCUG-ACCUCCTT-3′, was a gift of Dr. Clare Futter, Institute of Ophthalmology [32].

2.3. Transient knockdown of IQGAP1

The siRNAs were introduced into the cells by nucleofection (Amaxa). Controls either used the control oligonucleotide or mock transfections in which all the reagents except oligonucleotide were used during nucleofection. RBL-2H3 and K2 cells were passaged 2–3 days prior to nucleofection and were maintained in growth medium. General protocol for nucleofection of adherent cells was performed using solution R (Amaxa) and program T-20 for RBL-2H3, and solution NHDF and program U23 for K2.

2.4. Immunoblotting

To confirm knockdown lysates were prepared 48, 72 and 96 h post-nucleofection for analysis by western blotting. Equal amounts of total protein from the cell lysates were resolved by 10% SDS-PAGE before the transfer to nitrocellulose membranes. The membranes were blocked with blocking buffer (5% skim milk and 0.05% Tween in phosphate buffered saline) for 20 min before being probed with 1:1000 dilution of rabbit anti-IQGAP1 polyclonal antibody, directed against the $IQGAP1^{N}$ (N-terminal region, amino acids 1–863) [25]. The same membranes were also probed with antibodies