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Phospholipase Cγ negatively regulates Rac/Cdc42 activation in antigen-stimulated mast cells

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The Rho GTPases Rac and Cdc42 play a central role in the regulation of secretory and cytoskeletal responses in antigen-stimulated mast cells. In this study, we examine the kinetics and mechanism of Rac and Cdc42 activation in the rat basophilic leukemia RBL-2H3 cells. The activation kinetics of both Rac and Cdc42 show a biphasic profile, consisting of an early transient peak at 1 min and a late sustained activation phase at 20–40 min. The inhibition of phospholipase C (PLC)γ causes a twofold increase in Rac and Cdc42 activation that coincides with a dramatic production of atypical filopodia-like structures. Inhibition of protein kinase C using bisindolylmaleimide mimics the effect of PLCγ inhibition on Rac activation, but not on Cdc42 activation. In contrast, depletion of intracellular calcium leads to a complete inhibition of the early activation peak of both Rac and Cdc42, without significant effects on the late sustained activation. These data suggest that PLCγ is involved in a negative feedback loop that leads to the inhibition of Rac and Cdc42. They also suggest that the presence of intracellular calcium is a prerequisite for both Rac and Cdc42 activation.

Key words: Calcium · Cdc42 · Mast cells · Phospholipase C · Rac

Introduction

Mast cells participate in the pathogenesis of immediate hypersensitivity and allergic reactions in humans [1]. Moreover, mast cells are important contributors to airway hyper-responsiveness that occurs in asthmatic inflammation [2]. In response to allergens and other exogenous stimuli, mast cells accumulate in the airway smooth muscle and in the bronchial intraepithelial layer [3]. The activation of mast cells, through the cross-linking of the FcεRI, leads to the release of pre-formed early-phase inflammatory mediators and vasoactive substances from granules. It also leads to the *in situ* production of cytokines and bioactive lipids that contribute to the late-phase manifestation of the asthmatic reaction [4].

Aggregation of FcεRI on the surface of mast cells in response to multivalent ligand binding initiates a cascade of downstream signaling effectors that leads to degranulation [5]. These include phosphatidylinositol 3-kinase (PI3K), the guanine nucleotide exchange factor Vav, and phospholipase C (PLC)γ1 [6]. The early activation of PLCγ1 results in the generation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. These two second messengers lead to increases in cytoplasmic calcium and activation of calcium/diacylglycerol-regulated isoforms of protein kinase C (PKC) which culminate in granule margination and exocytosis [7]. PI3K plays an important regulatory role as an upstream activator of PLCγ1, as well as a regulator of calcium flux during mast cell degranulation [8–13].

Members of the Rho family of small GTPases also regulate signaling events in mast cells [14–16]. In the rat basophilic leukemia (RBL-2H3) cells, Rac and Cdc42 have been implicated in the control of the FcεRI-mediated phagocytosis and the regulation of migration,

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Abbreviations: **A-DNP:** albumin-dinitrophenol · **BAPTA-AM:** 1,2-bis-(2-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid acetoxymethyl ester · **CRIB:** Cdc42 and Rac interactive binding · **GAP:** GTPase-activating protein · **GEF:** guanine nucleotide exchange factor · **IP_3:** inositol 1,4,5-triphosphate · **PKC:** protein kinase C · **PLC:** phospholipase C · **RBL:** rat basophilic leukemia

as well as the induction of cytoskeletal rearrangements and exocytosis [17, 18]. Introduction of Rac into permeabilized mast cells causes exocytic secretion [19], and expression of dominant-negative forms of Rac and Cdc42 inhibits antigen-mediated degranulation [20]. This inhibition is rescued by the addition of calcium ionophores, implying that Rac and Cdc42 are upstream of the calcium influx pathway [21, 22]. Patch clamp studies in the same system showed that Rac/Cdc42 are involved in calcium mobilization, in part through the regulation of calcium release-activated calcium channels [23]. Rac/Cdc42 also play a positive role in the activation of PLCγ, the generation of IP_3, and the aggregation of the IP_3 receptor in response to the engagement of the FcεRI [21, 24].

In conjunction with the secretory response, antigen-stimulated mast cells undergo dramatic cytoskeletal responses and morphological changes [25]. These include the spreading of the cells on the substratum, formation of actin plaques on the basal surface of the cells, formation of lamellae on the apical surface, and the accumulation of actin into large cortical cables [25–28]. In mast cells, as in other systems, Rho family GTPases regulate the formation of these structures [20, 29–31]. The precise role of these actin structures with regards to degranulation is not yet understood, Numerous studies have suggested a negative role for actin with regard to degranulation [26, 29, 32–38] although this has been controversial [32].

The regulation of Rac and Cdc42 activation in RBL-2H3 cells is not well characterized. Previous studies have shown that the activation of Rho GTPases is PI3K-independent in RBL-2H3 cells [24]. PLCγ was found to directly bind Cdc42 *in vitro* in these cells [21], suggesting that PLCγ could be a downstream effector of Rho GTPases in response to antigen stimulation. In contrast, we have here examined the role of PLCγ in the upstream regulation of Rac and Cdc42. Using a well-established Rac/Cdc42 activation assay, we show that inhibition of PLCγ activates Rac and Cdc42 in RBL-2H3 cells. Regulation of Rac by PLCγ is mediated at least in part through PKC, whereas the regulation of Cdc42 is independent of PKC. We also show that the early but not late activation of Rac and Cdc42 is calcium-dependent. Our data suggest a novel role of PLCγ in modulating Rac and Cdc42 activation during antigen-mediated exocytosis in mast cells, and point to an unappreciated calcium-dependent mechanism of Rac and Cdc42 activation.

Results

Biphasic activation of Rac and Cdc42 in antigen-stimulated mast cells

We measured the activation of Rac and Cdc42 in antigen stimulated RBL-2H3 cells using a previously established Rac and Cdc42 activation assay [39]. The data are presented as a percentage of maximal stimulation, to facilitate a comparison of the time course, as well as in terms of fold stimulation, to compare the magnitude of

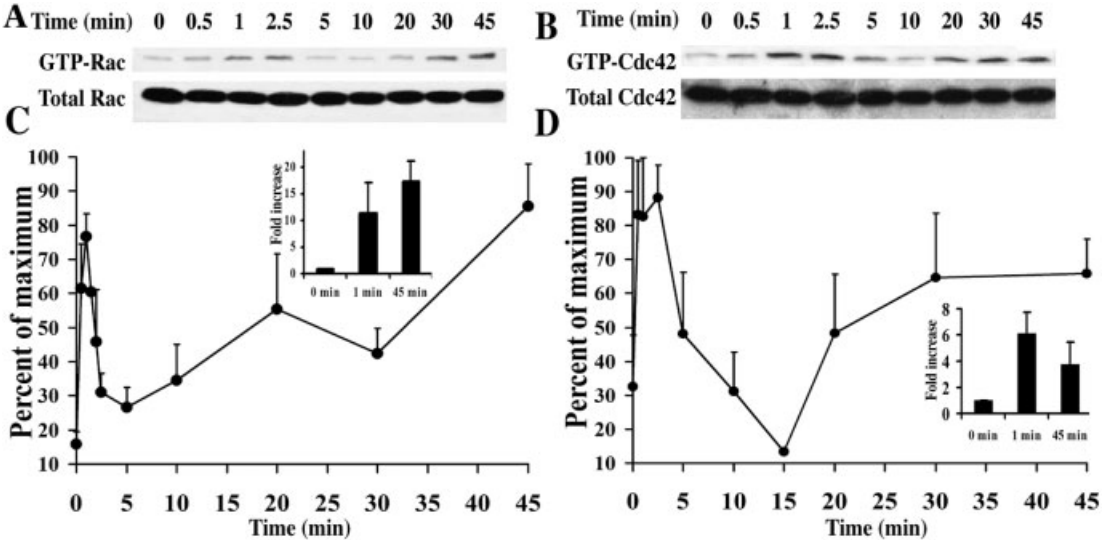

Figure 1. Rac and Cdc42 show a biphasic activation profile. (A, B) Representative Western blots of GST-CRIB pull-downs showing Rac and Cdc42 activation in antigen-stimulated RBL-2H3 cells. Proteins bound to immobilized GST-CRIB were immunoblotted with either anti-Rac (A) or anti-Cdc42 (B) antibodies. The lower gels show the amount of total Rac and Cdc42 in cell lysates. (C, D) Quantitation of the Western blot data by densitometry (arbitrary units) for Rac (C) and Cdc42 (D). Activation is calculated as percent of the maximum within each experiment. Insets: quantitation of Rac/Cdc42 activation, expressed as fold increase relative to basal. Data are the means ± SEM from six experiments for Rac and three experiments for Cdc42.

the response. Activation of both Rac and Cdc42 showed a biphasic profile, consisting of an early transient peak and a late persistent elevation of activity (Fig. 1A, B). As shown in Fig. 1C, Rac activation is detected as early as at 1 min. The activation drops to near basal levels by 5 min, but then increases again after 30–45 min. The magnitude of this second peak relative to the first is somewhat variable (see for example Fig. 2A). Like Rac, Cdc42 activation peaks at 1 min but returns to basal levels by 10 min, then increases again after 20 min (Fig. 1D). The early peak of Cdc42 activity is broader than that seen with Rac.

PLCγ inhibition increases the activation of Rac and Cdc42

Previously, we have shown that PI3K is essential for degranulation in antigen-responsive mast cells [11, 12]. However, it has been reported that Rac activation in response to antigen stimulation in mast cells is PI3K-independent [24]. This led us to examine other effectors in the degranulation pathway with regard to Rac and Cdc42 regulation. PLCγ is known to be an important mediator of the antigen response in mast cells. We tested the role of PLCγ in the regulation of Rac and Cdc42 using the specific PLCγ inhibitor U73122. The fold activation

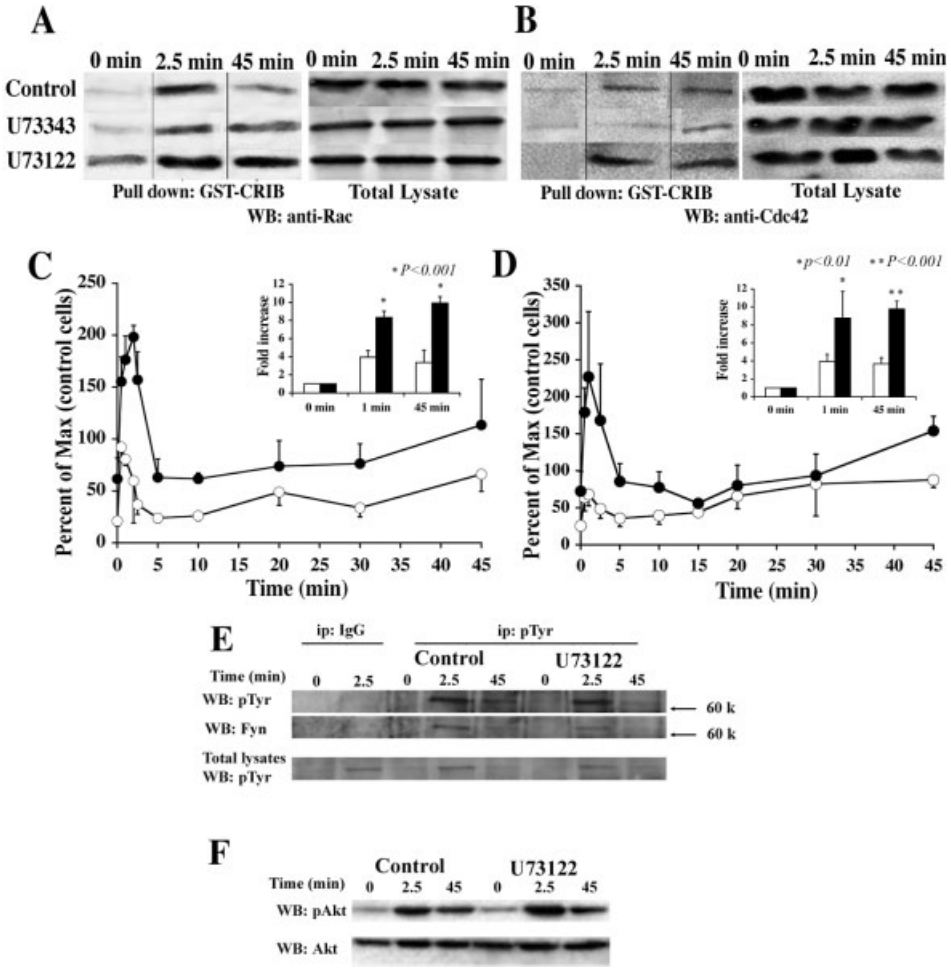

Figure 2. PLCγ inhibition enhances Rac and Cdc42 activation in antigen-stimulated cells. RBL-2H3 cells were treated with 5 μM U73122 or U73343 for 10 min at 37°C or left untreated. Cells were then stimulated with antigen for the indicated times and assayed for Rac and Cdc42 activation. (A, B) Representative Western blots of Rac and Cdc42 activation (left panels) or total levels of Rac and Cdc42 (right panels) in untreated or treated RBL-2H3 cells at 2.5 and 45 min after antigen stimulation. (C, D) Rac and Cdc42 activation in antigen-stimulated RBL-2H3 cells treated with U73122 (black circles and black bars) or left untreated (white circles and bars) was quantitated by densitometry. Activation is expressed as a percentage of maximum activity in untreated cells (line graph) or as fold increase over basal (inset). Data are the means ± SEM from four experiments for Rac and three experiments for Cdc42. Statistical significance was calculated using a one-tailed Student's *t*-test. (E) Cells were incubated in the absence or presence of U73122 and stimulated with antigen for various times. Anti-phosphotyrosine (and anti-IgG for control) immunoprecipitates were blotted with anti-pTyr and anti-Fyn antibodies. The total cell lysates were also blotted with anti-pTyr antibody. (F) Cells were incubated in the absence or presence of U73122, stimulated with antigen for various times, and lysates were blotted with anti-p473-Akt and Akt antibodies.

of Rac and Cdc42 in control cells was lower in this experiment as compared to Fig. 1, reflecting variability in the magnitude of the mast cell response to antigen.

Nonetheless, inhibition of PLCγ led to a significant increase in the activation of both Rac and Cdc42 (Fig. 2A, B). In cells treated with U73122, Rac and Cdc42 activation was at least twofold elevated relative to the untreated cells at both early and late time points (Fig. 2C, D). However, PLCγ inhibition did not alter the kinetics of Rac and Cdc42 activation, which still showed distinct early and late peaks of activity. In contrast, treatment with the inactive isoform of the inhibitor, U73343, caused a slight decrease in Rac and Cdc42 activation (Fig. 2A, B), presumably due to the effects of the DMSO carrier. Inhibition of PLCγ had no effect on antigen stimulation of other early signaling events in these cells, such as activation of Fyn (Fig. 2E); anti-phosphotyrosine immunoprecipitates from both control or drug-treated cells contained a 60-kDa band that was recognized by both anti-Fyn and anti-phosphotyrosine antibodies. Similarly, activation of the PI3K pathway, as detected by phosphorylation of the downstream effector Akt, was unaffected by treatment of cells with U73122 (Fig. 2F).

PLCγ regulates actin in antigen-stimulated RBL-2H3 cells

Rac and Cdc42 regulate actin responses during the activation of RBL-2H3 cells [20]. Having found that PLCγ inhibition leads to an increase in Rac and Cdc42 activation, we next tested the effect of PLCγ inhibition on the actin rearrangements that occur in response to antigen. RBL-2H3 cells were treated without or with U73122 or U73343, stimulated for 2.5 or 45 min, and fixed and stained with fluorescent phalloidin.

RBL-2H3 cells treated with the inactive isoform of the PLCγ inhibitor (U73343) showed the same actin response as untreated cells (Fig. 3, upper and middle panels). After 2.5 min of stimulation, the cells showed an increase in surface area, with the formation of membrane ruffles and peripheral actin cables and the appearance of actin plaques at the substratum; these changes were variably evident after 45 min of stimulation (Fig. 3, upper panel). In contrast, cells treated with U73122 showed a marked antigen-stimulated increase in peripheral actin-rich structures that resembled filopodia (Fig. 3, lower panel). The cells also showed disappearance of the actin cables at the periphery and actin plaques at the substratum. Given the involvement of Rac and Cdc42 in the formation of filopodia in other systems [29], these data suggest that PLCγ plays a role in negatively regulating Rac and Cdc42 activity and Rac/Cdc42-dependent actin rearrangements in antigen-stimulated mast cells.

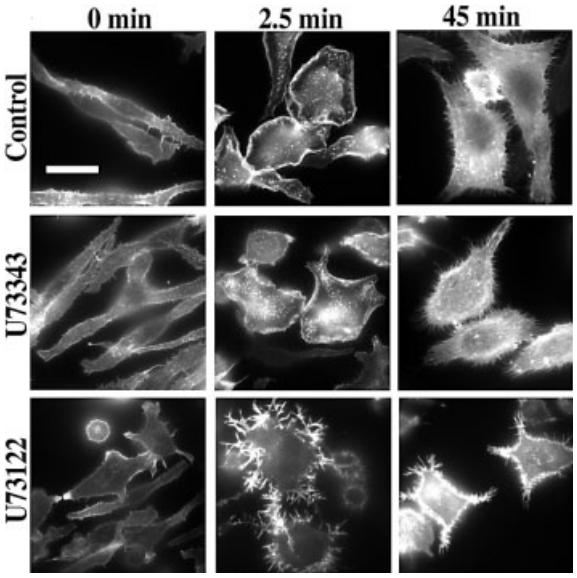

Figure 3. PLCγ inhibition leads to the formation of filopodia-like structures in antigen-stimulated RBL-2H3 cells. Representative fluorescent micrographs of RBL-2H3 cells stained with rhodamine phalloidin to visualize F-actin. Cells were left untreated (upper panels) or treated with 5 μM of U73343 (middle panels) or U73122 (lower panels) for 10 min at 37°C. The cells were stimulated with antigen for various times and fixed. Scale bar is 10 μm.

Inhibition of PKC mimics the effect of PLCγ inhibition

PLCγ hydrolyses phosphatidylinositol 4,5-bisphosphate, leading to the production of diacylglycerol and IP_3. These products lead respectively to the activation of cPKC and nPKC and to the release of calcium from internal stores. To determine which downstream effector(s) are responsible for mediating the negative effect of PLCγ on Rac and Cdc42, we first examined whether inhibition of PKC would mimic the effects of PLCγ inhibition. RBL-2H3 cells were treated with 400 nM of the PKC inhibitor bisindolylmaleimide. This resulted in an increase in antigen-stimulated Rac activation at all times as compared to control cells (Fig. 4A). This increase was most dramatic at 1 min (2.7-fold), whereas effects at late time points (30–45 min) were not statistically significant. In contrast, PKC inhibition had no effect on Cdc42 activation (Fig. 4B).

We also examined the effect of PKC inhibition on antigen-stimulated actin responses in RBL-2H3 cells. Treatment of cells with bisindolylmaleimide partially mimicked the effect of PLCγ inhibition, leading to a loss of peripheral actin cables and an increase in filopodia-like structures (Fig. 5). The increase in filopodia was less dramatic in bisindolylmaleimide-treated cells as compared to those treated with the PLCγ inhibitor. Furthermore, unlike U73122-treated cells, the bisindo-

lylmaleimide-treated cells showed actin plaque formation at 2.5 min. This partial response could be explained by the fact that PKC inhibition led to increased Rac activation but had no effect on Cdc42 activation.

PLC and PKC regulate Vav translocation to the membrane

In RBL-2H3 cells, it has been shown that Vav translocation reflects its activation [40]. In order to explore the mechanism by which PLCγ and PKC regulate Rac and Cdc42, we looked at the effect of PLCγ and PKC inhibition on Vav translocation in RBL-2H3 cells. In unstimulated cells and antigen-stimulated control cells, anti-Vav staining was diffuse and did not clearly label distinct subcellular structures (Fig. 6A, B). Treatment of unstimulated cells with either inhibitor had no effect of Vav localization (Fig. 6D, G). In antigen-stimulated cells treated with U73122 or bisindolylmaleimide, the production of filopodia-like structure was less pronounced than in Fig. 3 (Fig. 6F, I). Nonetheless, antigen stimulation caused a marked increase in membrane-associated Vav in cells treated with the inhibitors (Fig. 6E, H). These data suggest that in control cells, antigen-stimulated activation of PLC and PKC suppresses Vav activation.

Role of calcium in the activation of Rac and Cdc42 in response to antigen

Since the negative regulation of Cdc42 was not dependent on PKC, we tested the possibility that PLCγ could be mediating its effect on Cdc42 through calcium. We treated the cells with 10 μm of the calcium chelator 1,2-bis-(2-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM), in order to inhibit both intracellular and store-operated calcium replenishment. Surprisingly, we found that calcium depletion abolished the early activation peak of both Rac and Cdc42 (Fig. 7A, B). In contrast, the late activation of Rac and Cdc42 (at 20–45 min) appeared to be calcium-independent. The failure of BAPTA to inhibit the late phase of Rac/Cdc42 was not due to a loss of drug potency, as pre-incubation of cells with BAPTA for 45 min prior to EGF stimulation produced identical results, with preferential inhibition of the early activation phase (Fig. 7C). Calcium depletion in these cells also led to a decrease in total actin in the cells and to the disappearance of actin structures in response to antigen

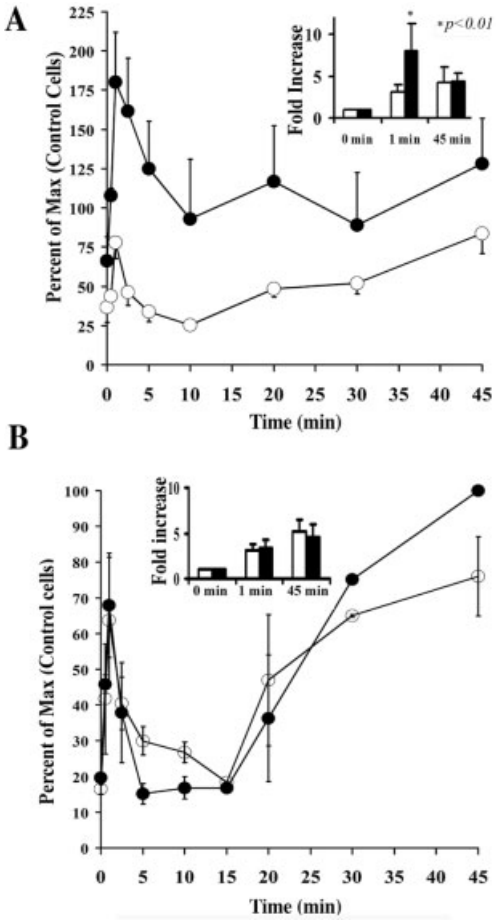

Figure 4. PKC inhibition mimics the effect of PLCγ inhibition on Rac but not Cdc42. RBL-2H3 cells were incubated in the absence or presence of 400 nM bisindolylmaleimide for 15 min and stimulated with antigen for the indicated times. Rac (A) and Cdc42 (B) activity was measured as in Fig. 1. Activation is expressed as percentage of maximum activation in untreated cells or as fold increase over basal (insets) in each experiment; bisindolylmaleimide: black circles and black bars; no treatment: white circles and bars. The data are the means ± SEM from six experiments for Rac and three experiments for Cdc42. Statistical significance was calculated using a one-tailed Student's *t*-test.

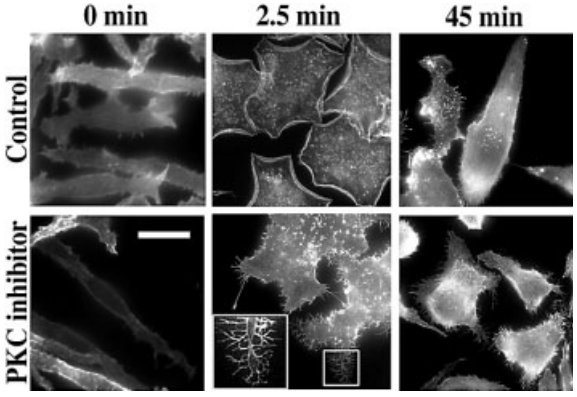

Figure 5. PKC inhibition partially mimics the effect of PLCγ inhibition on actin in RBL-2H3 cells. Cells were incubated in the absence (upper panels) or presence (lower panels) of 400 nM of bisindolylmaleimide for 15 min. The cells were then stimulated with antigen for the indicated times, fixed, and stained with rhodamine phalloidin. Scale bar is 10 μm.

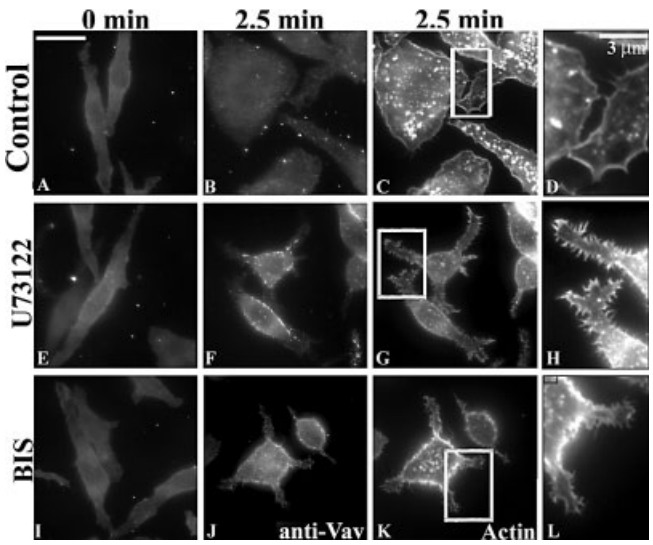

Figure 6. Inhibition of PLCγ and PKC induces the membrane translocation of Vav translocation. RBL-2H3 cells were incubated in the absence (A–D) or presence of U73122 (E–H) or bisindolylmaleimide (I–L) as indicated. The cells were stimulated with antigen for various times, fixed, and stained with anti-Vav antibody (A, B, E, F, I, J) or rhodamine phalloidin (C, D, G, H, K, L). Scale bar is 10 μm; (D, H, L) are magnifications of the indicated areas in (C, H, K).

stimulation (data not shown). Conversely, Rac was constitutively activated in cells treated for 10 min with the calcium ionophore ionomycin (Fig. 7D), which also stimulated degranulation (data not shown). Taken together, these data show that calcium flux is both necessary and sufficient to induce Rac activation.

Discussion

Cross-linking of the IgE-bound high-affinity receptor FcεRI with polyvalent antigen leads to calcium-dependent degranulation in mast cells [25]. Based on studies using dominant-negative or activated mutants, Rho GTPases appear to be central to the signaling cascades that govern the biochemical and cytoskeletal changes required for mast cell degranulation [19]. In this report, we examine the regulation of endogenous Rac and Cdc42 in mast cells. We find that both Rac and Cdc42 follow a biphasic activation profile, with an early peak of activation at 1 min and a late phase of activation that was sustained up to 45 min. Surprisingly, we find that PLCγ inhibition leads to an increase in Vav localization to the plasma membrane, an increase in Rac and Cdc42 activation, and a production of atypical filopodia-like structures. These responses are partially mediated by

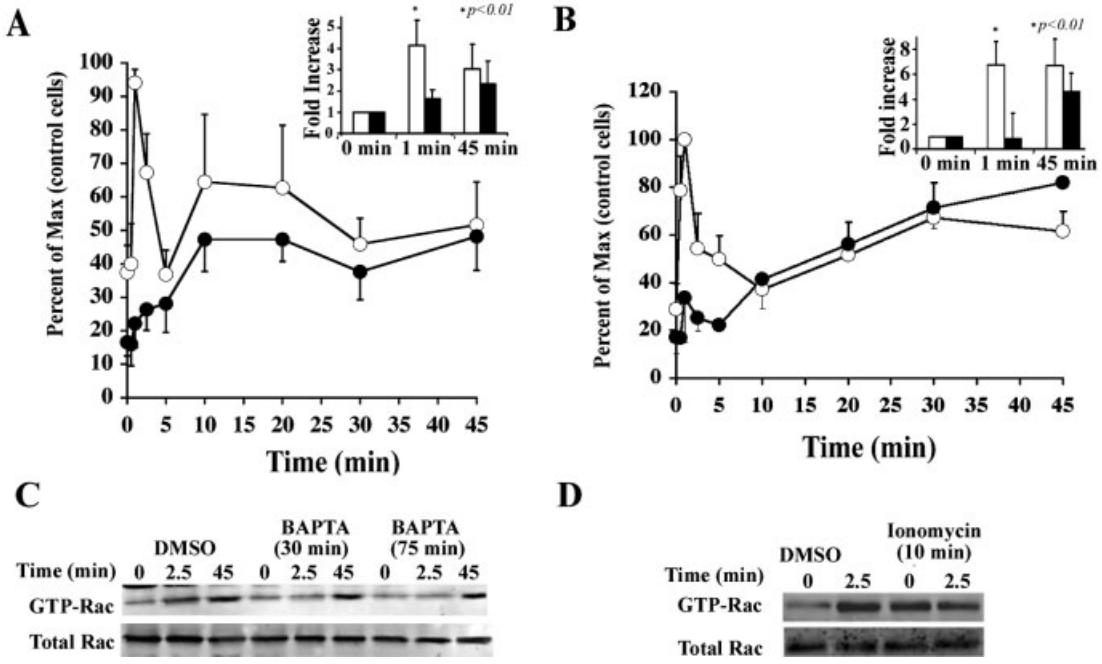

Figure 7. The early peak of antigen-stimulated Rac and Cdc42 activity is calcium-dependent. RBL-2H3 cells were incubated in the absence or presence of 10 μM BAPTA-AM for 30 min, and then assayed for activation of Rac (A) and Cdc42 (B) as in Fig. 1. Activation is expressed as percentage of maximum activation in untreated cells or as fold increase over basal (insets) in each experiment; BAPTA: black circles and black bars; no treatment: white circles and bars. Data are the means ± SEM from five experiments for Rac and three experiments for Cdc42. (C) Cells were treated with BAPTA for 30 or 75 min and then assayed for Rac activation by GST-CRIB pull-down (upper gel). The lower gel is a Western blot showing the total amount of Rac in the cells. (D) Cells were treated with ionomycin (1 μM for 15 min) or left untreated, then stimulated with antigen for 2.5 min. Rac activation was measured by GST-CRB pull-down.

inhibition of PKC. Our data suggest a novel negative feedback loop, in which antigen-stimulated activation of PLCγ leads to an attenuation of Rac and Cdc42 activation.

Interestingly, previous studies have shown that Rac and Cdc42 are upstream activators of PLCγ in RBL-2H3 cells. Rac and Cdc42 are believed to regulate the phosphorylation and activation of PLC, and PLCγ directly binds to activated Cdc42 *in vitro* [21, 24]. Furthermore, tyrosine phosphorylation of PLCγ is abrogated in response to Toxin B treatment and in bone marrow mast cells from $Vav^{-/-}$ mice [41, 42]. Patch clamp studies have established that Rac and Cdc42 regulate the early calcium release-activated calcium current (I_{CRAC}) as well as receptor-mediated calcium influx [23]. Expression of constitutively active mutants of Rac and Cdc42 in B6A4C1 cells, which are deficient in the calcium response, reconstituted the early (1 min) calcium influx wave [22, 43]. Taken together, these observations suggest that Rac and Cdc42 are most likely upstream of PLCγ. In this light, our finding that PLCγ inhibits Rac and Cdc42 suggests a classical negative feedback loop, in which PLCγ negatively regulates the activation of Rac and Cdc42 and terminates or limits the secretory response.

The inhibition of PLCγ led to a twofold increase in Rac and Cdc42 activation. This effect was not mediated by increase in PI3K activation, since PLCγ inhibition did not have a significant effect on the phosphorylation of Akt. PLCγ inhibition enhanced the membrane translocation of Vav in response to antigen, suggesting that PLCγ inhibits Cdc42 and Rac at least in part by negatively regulating Vav. However, inhibition of PKC with bisindolylmaleimide also enhanced antigen-stimulated Vav translocation yet had no effect on Cdc42, suggesting that this cannot be sufficient for Cdc42 activation. Another possible effector of PLCγ in RBL-2H3 cells is β2-chimaerin, which is a diacylglycerol-stimulated GTPase-activating protein (GAP) specific for Rac [44]. A recent study in HeLa cells in fact has shown that activation of β-chimaerin inhibits EGF stimulation of Rac [45]. This mechanism could explain the effects of PLCγ on Rac in mast cells. In contrast, DAG-regulated GAP for Cdc42 have not yet been described, although the existence of such an enzyme is plausible.

We also looked at PKC downstream from PLC. We find that Rac, but not Cdc42, is activated by inhibition of PKC, suggesting that Rac and Cdc42 are differentially regulated in response to antigen stimulation. Studies in bone marrow mast cells derived from $PKC\delta^{-/-}$ mice showed that PKCδ plays a negative role in calcium mobilization and antigen-induced mast cell degranulation [46]. Given that Rac and Cdc42 activation are required for calcium signaling in mast cells [21–24, 43], the effects of PKCδ on degranulation could be in part *via* inhibition of Rac. The mechanism for PKC-mediated inhibition of Rac is yet to be determined but could involve PKC regulation of a Rac-specific guanine nucleotide exchange factor (GEF) or GAP, analogous to the inhibition of Ral-GEF by PKC [47]. Interestingly, Parekh *et al*. [48] have shown that PKC negatively regulates the calcium release-activated calcium influx pathway (I_{CRAC}) in RBL-2H3 cells. Given that treatment of cells with a calcium ionophore is sufficient for the activation of Rac, inhibition of PKC could activate Rac through an increase in I_{CRAC}-mediated calcium influx. However, it is not clear why Cdc42 would not be activated under these conditions.

In RBL-2H3 cells, the early peak of activation of both Rac and Cdc42 is dependent on intracellular calcium. This suggests the possible involvement of a calcium-dependent Rac/Cdc42 GEF. Although calcium-regulated GEF for Ras, R-Ras and Rap-1 have been identified [49, 50], a calcium-dependent Rac/Cdc42-GEF has not yet been described. Alternatively, the calcium-dependent phosphorylation of RhoGDIα leads to Rac membrane translocation and activation in PC3 human prostrate cells [51]. A similar mechanism might also regulate Rac and Cdc42 in mast cells.

The calcium dependence of Rac and Cdc42 suggests the mobilization or utilization of a PLCγ-independent calcium pool. There are several calcium mobilization responses that have been isolated in antigen-stimulated RBL-2H3 cells, only a subset of which are mediated by PLCγ. This could explain the presence of a calcium-dependent early Rac and Cdc42 activation phase, even in the presence of the PLCγ inhibitor. Work from our laboratory and others showed a requirement for PI3K in mast cell degranulation and calcium influx [8, 10, 12], and PLCγ-independent phosphatidylinositol 3,4,5-triphosphate-sensitive calcium channels at the plasma membrane have been characterized [9]. However, PI3K is not required for activation of Rac and Cdc42 in mast cells [10, 13, 24], where it may in fact be downstream of Rac [24, 52]. Alternatively, sphingosine kinase-1 mediates fast and transient calcium release from internal stores [53] and has been implicated in Rac and Cdc42 activation in other systems [54, 55]. Sphingosine kinase-1 is a potential regulator of the calcium pools required for the early activation of Rac and Cdc42 in RBL-2H3 cells.

Finally, inhibition of PLCγ leads to a change in actin responses that parallels the increases in Rac and Cdc42 activity. Normally, increases in Rac and Cdc42 activation would be predicted to cause an increase in protrusion, ruffling, and actin plaque formation at the substratum [20]. Instead, we find that the increases in Rac/Cdc42 activity caused by inhibition of PLCγ lead to the appearance of atypical filopodia-like structures. These filopodia-like structures are not caused by inhibition of

PLCγ per se, as they are only seen after antigen stimulation. They therefore represent a derangement of the normal cellular response to antigen.

Filopodia are formed as the result of a failure in the formation of a branched actin network, due to the decrease in actin filament severing or the increase in free barbed end capping [56]. Some actin severing proteins, including gelsolin, are regulated by PLCγ [57]. In many cell systems, gelsolin promotes actin polymerization by creating new barbed ends at the cell periphery [30], and gelsolin is an important regulator of the actin structures in RBL-2H3 cells [58]. An inhibition of PLCγ-mediated gelsolin activation could decrease the production of free barbed ends needed for a branched actin network [59], leading to the production of filopodia-like actin structures.

In summary, we have shown that activation of Rac and Cdc42 in antigen-stimulated mast cells is negatively regulated through PLCγ. The increased activation of Rac/Cdc42 is coincident with enhanced production of filopodia-like structures. PLCγ inhibition of Rac, but not Cdc42, works at least in part through PKC. Further characterization of this novel negative feedback pathway will have important implications for understanding antigen-stimulated exocytosis in mast cells.

Materials and methods

Cell culture

RBL-2H3 cells [60] were cultured in IMDM containing 15% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37°C. Cells were either plated in Nunc tissue culture dishes or on 18-mm glass coverslips coated with 10 μg/mL Fibronectin.

Antibodies and reagents

Mouse anti-Rac monoclonal antibody (clone 23A8) was obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-Cdc42 polyclonal antibody (sc-87) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Vav, rabbit anti-Fyn, rabbit anti-Akt, and mouse anti-phospho-Akt (Ser 473) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal mouse anti-dinitrophenol (DNP) IgE (cl-SPE-7), DNP-conjugated albumin (A-DNP), wortmannin, ionomycin, and BAPTA were obtained from Sigma (St. Louis, MO). U73122, U73343 and bisindolylmaleimide I were obtained from Calbiochem-Novabiochem Co. (San Diego, CA). HRP-coupled anti-mouse and anti-rabbit IgG were from Bio-Rad. ECL detection reagents were purchased from Amersham Corp. (Arlington Heights, IL) and rhodamine phalloidin was obtained from Molecular Probes (Eugene, OR). The GST-Cdc42 and Rac interactive binding (CRIB) construct was a generous gift from Dr. Klaus Hahn, University of North Carolina.

Inhibitor experiments

In all experiments, cells were incubated overnight with 0.1 μg/mL anti-DNP IgE. The cells were washed with Hanks' balanced salt solution (HBSS) and then stimulated with HBSS/1 mM calcium containing 100 ng/mL A-DNP. In calcium depletion experiments, the stimulation medium was calcium-free. When indicated, BAPTA-AM was added to the culture media to a final concentration of 10 μM for 30 min before stimulation. Cells were then washed with HBSS and stimulated with HBSS containing A-DNP and BAPTA-AM. For experiments using inhibitors, the cells were pre-incubated with HBSS/calcium at 37°C in the absence or presence of U73122 (5 μM for 10 min), U73343 (5 μM for 10 min), bisindolylmaleimide (400 nM for 15 min), wortmannin (100 nM for 15 min), or ionomycin (1 μM for 2 h) as indicated. The cells were then stimulated with A-DNP for various times.

Protein preparation and Rac activation assay

Activation of Rac and Cdc42 was assayed using the CRIB domain of PAK (residues 56–272). GST-CRIB was expressed in *Escherichia* coli and purified using glutathione-agarose. Anti-DNP IgE-preloaded RBL-2H3 cells were stimulated with A-DNP for various times, then stopped by the addition of ice-cold PBS containing 1 mM sodium orthovanadate. The cells were lysed in buffer A (25 mM HEPES, 1% Igepal, 150 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 1 mM $NaVO_4$, 20 mM NaF, 1 mM PMSF, 100 μg/mL aprotinin, and 5 μM leupeptin). Lysates were cleared by centrifugation and incubated with the GST-CRIB protein (20 μg) for 30 min at 4°C while rotating. The beads were washed three times with buffer A and boiled in Laemmli sample buffer. Rac and Cdc42 were detected by Western blotting using the anti-Rac and Cdc42 antibodies and ECL detection, and quantitated by densitometry using the NIH image 1.62 analysis software.

Immunoprecipitation

RBL-2H3 cells were lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EGTA, 5 mM sodium pyrophosphate, 30 mM β-glycerolphosphate, 30 mM sodium fluoride, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM sodium orthovanadate, 2 μg/mL leupeptin, 4 μg/mL pepstatin, and 0.5% Triton X-100) after drug treatment and stimulation and centrifuged at 14 000 rpm at 4°C for 20 min. For each immunoprecipitation, the supernatants were incubated with 5 μg of the monoclonal anti-phosphotyrosine antibody PY-20 or mouse IgG1 antibody as a control. The samples were mixed overnight, and then 20 μL of resuspended protein-G-agarose was added, and the samples were rocked for 1 h. The samples were separated by 10% gradient SDS-PAGE.

Degranulation assays

RBL-2H3 cells were incubated overnight in 0.1 μg/mL anti-DNP IgG. The cells were washed in HBSS and stimulated for 45 min at 37°C with HBSS/1 mM calcium containing 10 ng/mL A-DNP. The supernatant was then removed and brought to 100 mM citrate, pH 4.5, 1 mM 4-methylumbelliferyl-N-acetyl

glucosamine (Sigma). After 15 min at 37°C, the reaction was stopped with 1/10 volume of 200 mM Na_2CO_3, glycine, pH 10.7, and substrate hydrolysis measured using a fluorescence spectrophotometer (360 excitation/465 emission).

Immunostaining and microscopy

Cells plated on coverslips were stimulated for various times and fixed with 4% paraformaldehyde at 37°C. The cells were permeabilized and incubated with rhodamine phalloidin in 1% BSA for 2 h at room temperature. Cells were alternatively immunostained with primary antibody and then incubated with Alexa 488-conjugated secondary antibody (Molecular Probes). The cells were visualized on a Nikon Eclipse 400 microscope using a 60× 1.4 N.A. plan-apo infinity-corrected objective and the images were collected using a Roper cooled CCD camera.

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References

1 **Marshall, G. D.,** Internal and external environmental influences in allergic diseases. *J. Am. Osteopath. Assoc.* 2004. **104:** S1–S6.

2 **Robinson, D. S.,** The role of the mast cell in asthma: Induction of airway hyperresponsiveness by interaction with smooth muscle? *J. Allergy Clin. Immunol.* 2004. **114:** 58–65.

3 **Olsson, N., Ulfgren, A. K. and Nilsson, G.,** Demonstration of mast cell chemotactic activity in synovial fluid from rheumatoid patients. *Ann. Rheum. Dis.* 2001. **60:** 187–193.

4 **Bingham, C. O., 3rd and Austen, K. F.,** Mast-cell responses in the development of asthma. *J. Allergy Clin. Immunol.* 2000. **105:** S527–S534.

5 **Hamawy, M. M., Mergenhagen, S. E. and Siraganian, R. P.,** Protein tyrosine phosphorylation as a mechanism of signalling in mast cells and basophils. *Cell. Signal.* 1995. **7:** 535–544.

6 **Turner, H. and Kinet, J. P.,** Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature* 1999. **402:** B24–B30.

7 **Katakami, Y., Kaibuchi, K., Sawamura, M., Takai, Y. and Nishizuka, Y.,** Synergistic action of protein kinase C and calcium for histamine release from rat peritoneal mast cells. *Biochem. Biophys. Res. Commun.* 1984. **121:** 573–578.

8 **Barker, S. A., Lujan, D. and Wilson, B. S.,** Multiple roles for PI 3-kinase in the regulation of PLCgamma activity and Ca^{2+} mobilization in antigen-stimulated mast cells. *J. Leukoc. Biol.* 1999. **65:** 321–329.

9 **Ching, T. T., Hsu, A. L., Johnson, A. J. and Chen, C. S.,** Phosphoinositide 3-kinase facilitates antigen-stimulated Ca(2+) influx in RBL-2H3 mast cells *via* a phosphatidylinositol 3,4,5-trisphosphate-sensitive Ca(2+) entry mechanism. *J. Biol. Chem.* 2001. **276:** 14814–14820.

10 **Laffargue, M., Calvez, R., Finan, P., Trifilieff, A., Barbier, M., Altruda, F., Hirsch, E. and Wymann, M. P.,** Phosphoinositide 3-kinase gamma is an essential amplifier of mast cell function. *Immunity* 2002. **16:** 441–451.

11 **Smith, A. J., Surviladze, Z., Gaudet, E. A., Backer, J. M., Mitchell, C. A. and Wilson, B. S.,** p110beta and p110delta phosphatidylinositol 3-kinases up-regulate Fc(epsilon)RI-activated Ca^{2+} influx by enhancing inositol 1,4,5-trisphosphate production. *J. Biol. Chem.* 2001. **276:** 17213–17220.

12 **Windmiller, D. A. and Backer, J. M.,** Distinct phosphoinositide 3-kinases mediate mast cell degranulation in response to G-protein-coupled *versus* FcepsilonRI receptors. *J. Biol. Chem.* 2003. **278:** 11874–11878.

13 **Barker, S. A., Caldwell, K. K., Pfeiffer, J. R. and Wilson, B. S.,** Wortmannin-sensitive phosphorylation, translocation, and activation of PLCgamma1, but not PLCgamma2, in antigen-stimulated RBL-2H3 mast cells. *Mol. Biol. Cell* 1998. **9:** 483–496.

14 **Pinxteren, J. A., O'Sullivan, A. J., Larbi, K. Y., Tatham, P. E. and Gomperts, B. D.,** Thirty years of stimulus-secretion coupling: From Ca(2+) to GTP in the regulation of exocytosis. *Biochimie* 2000. **82:** 385–393.

15 **Pinxteren, J. A., O'Sullivan, A. J., Tatham, P. E. and Gomperts, B. D.,** Regulation of exocytosis from rat peritoneal mast cells by G protein beta gamma-subunits. *EMBO J.* 1998. **17:** 6210–6218.

16 **Howell, T. W., Cockcroft, S. and Gomperts, B. D.,** Essential synergy between Ca^{2+} and guanine nucleotides in exocytotic secretion from permeabilized rat mast cells. *J. Cell Biol.* 1987. **105:** 191–197.

17 **Massol, P., Montcourrier, P., Guillemot, J. C. and Chavrier, P.,** Fc receptor-mediated phagocytosis requires CDC42 and Rac1. *EMBO J.* 1998. **17:** 6219–6229.

18 **Woo, C. H., Jeong, D. T., Yoon, S. B., Kim, K. S., Chung, I. Y., Saeki, T. and Kim, J. H.,** Eotaxin induces migration of RBL-2H3 mast cells *via* a Rac-ERK-dependent pathway. *Biochem. Biophys. Res. Commun.* 2002. **298:** 392–397.

19 **Brown, A. M., O'Sullivan, A. J. and Gomperts, B. D.,** Induction of exocytosis from permeabilized mast cells by the guanosine triphosphatases Rac and Cdc42. *Mol. Biol. Cell* 1998. **9:** 1053–1063.

20 **Guillemot, J. C., Montcourrier, P., Vivier, E., Davoust, J. and Chavrier, P.,** Selective control of membrane ruffling and actin plaque assembly by the Rho GTPases Rac1 and CDC42 in FcepsilonRI-activated rat basophilic leukemia (RBL-2H3) cells. *J. Cell Sci.* 1997. **110:** 2215–2225.

21 **Hong-Geller, E. and Cerione, R. A.,** Cdc42 and Rac stimulate exocytosis of secretory granules by activating the IP(3)/calcium pathway in RBL-2H3 mast cells. *J. Cell Biol.* 2000. **148:** 481–494.

22 **Hong-Geller, E., Holowka, D., Siraganian, R. P., Baird, B. and Cerione, R. A.,** Activated Cdc42/Rac reconstitutes Fcepsilon RI-mediated Ca^{2+} mobilization and degranulation in mutant RBL mast cells. *Proc. Natl. Acad. Sci. USA* 2001. **98:** 1154–1159.

23 **Djouder, N., Prepens, U., Aktories, K. and Cavalie, A.,** Inhibition of calcium release-activated calcium current by Rac/Cdc42-inactivating clostridial cytotoxins in RBL cells. *J. Biol. Chem.* 2000. **275:** 18732–18738.

24 **Djouder, N., Schmidt, G., Frings, M., Cavalie, A., Thelen, M. and Aktories, K.,** Rac and phosphatidylinositol 3-kinase regulate the protein kinase B in Fc epsilon RI signaling in RBL 2H3 mast cells. *J. Immunol.* 2001. **166:** 1627–1634.

25 **Oliver, J. M., Seagrave, J., Stump, R. F., Pfeiffer, J. R. and Deanin, G. G.,** Signal transduction and cellular response in RBL-2H3 mast cells. *Prog. Allergy* 1988. **42:** 185–245.

26 **Frigeri, L. and Apgar, J. R.,** The role of actin microfilaments in the down-regulation of the degranulation response in RBL-2H3 mast cells. *J. Immunol.* 1999. **162:** 2243–2250.

27 **Ludowyke, R. I., Kawasugi, K. and French, P. W.,** PMA and calcium ionophore induce myosin and F-actin rearrangement during histamine secretion from RBL-2H3 cells. *Cell Motil. Cytoskeleton* 1994. **29:** 354–365.

28 **Holst, J., Sim, A. T. and Ludowyke, R. I.,** Protein phosphatases 1 and 2A transiently associate with myosin during the peak rate of secretion from mast cells. *Mol. Biol. Cell* 2002. **13:** 1083–1098.

29 **Hall, A.,** Rho GTPases and the actin cytoskeleton. *Science* 1998. **279:** 509–514.

30 **Condeelis, J.,** How is actin polymerization nucleated *in vivo*? *Trends Cell Biol.* 2001. **11:** 288–293.

31 **Sullivan, R., Price, L. S. and Koffer, A.,** Rho controls cortical F-actin disassembly in addition to, but independently of, secretion in mast cells. *J. Biol. Chem.* 1999. **274:** 38140–38146.

32 **Pendleton, A. and Koffer, A.,** Effects of latrunculin reveal requirements for the actin cytoskeleton during secretion from mast cells. *Cell Motil. Cytoskeleton* 2001. **48:** 37–51.

33 **Koffer, A., Tatham, P. E. and Gomperts, B. D.,** Changes in the state of actin during the exocytotic reaction of permeabilized rat mast cells. *J. Cell Biol.* 1990. **111:** 919–927.

34 **Holowka, D., Sheets, E. D. and Baird, B.,** Interactions between Fc(epsilon)RI and lipid raft components are regulated by the actin cytoskeleton. *J. Cell Sci.* 2000. **113:** 1009–1019.

35 **Muallem, S., Kwiatkowska, K., Xu, X. and Yin, H. L.,** Actin filament disassembly is a sufficient final trigger for exocytosis in nonexcitable cells. *J. Cell Biol.* 1995. **128:** 589–598.

36 **Seagrave, J., Pfeiffer, J. R., Wofsy, C. and Oliver, J. M.,** Relationship of IgE receptor topography to secretion in RBL-2H3 mast cells. *J. Cell. Physiol.* 1991. **148:** 139–151.

37 **Seagrave, J. and Oliver, J. M.,** Antigen-dependent transition of IgE to a detergent-insoluble form is associated with reduced IgE receptor-dependent secretion from RBL-2H3 mast cells. *J. Cell. Physiol.* 1990. **144:** 128–136.

38 **Gimborn, K., Lessmann, E., Kuppig, S., Krystal, G. and Huber, M.,** SHIP down-regulates FcepsilonR1-induced degranulation at supraoptimal IgE or antigen levels. *J. Immunol.* 2005. **174:** 507–516.

39 **Benard, V. and Bokoch, G. M.,** Assay of Cdc42, Rac, and Rho GTPase activation by affinity methods. *Methods Enzymol.* 2002. **345:** 349–359.

40 **Arudchandran, R., Brown, M. J., Peirce, M. J., Song, J. S., Zhang, J., Siraganian, R. P., Blank, U. and Rivera, J.,** The Src homology 2 domain of Vav is required for its compartmentation to the plasma membrane and activation of c-Jun NH(2)-terminal kinase 1. *J. Exp. Med.* 2000. **191:** 47–60.

41 **Guinamard, R., Aspenstrom, P., Fougereau, M., Chavrier, P. and Guillemot, J. C.,** Tyrosine phosphorylation of the Wiskott–Aldrich syndrome protein by Lyn and Btk is regulated by CDC42. *FEBS Lett.* 1998. **434:** 431–436.

42 **Manetz, T. S., Gonzalez-Espinosa, C., Arudchandran, R., Xirasagar, S., Tybulewicz, V. and Rivera, J.,** Vav1 regulates phospholipase Cgamma activation and calcium responses in mast cells. *Mol. Cell. Biol.* 2001. **21:** 3763–3774.

43 **Field, K. A., Apgar, J. R., Hong-Geller, E., Siraganian, R. P., Baird, B. and Holowka, D.,** Mutant RBL mast cells defective in Fc epsilon RI signaling and lipid raft biosynthesis are reconstituted by activated Rho-family GTPases. *Mol. Biol. Cell* 2000. **11:** 3661–3673.

44 **Caloca, M. J., Wang, H. and Kazanietz, M. G.,** Characterization of the Rac-GAP (Rac-GTPase-activating protein) activity of beta2-chimaerin, a 'non-protein kinase C' phorbol ester receptor. *Biochem. J.* 2003. **375:** 313–321.

45 **Wang, H., Yang, C., Leskow, F. C., Sun, J., Canagarajah, B., Hurley, J. H. and Kazanietz, M. G.,** Phospholipase Cgamma/diacylglycerol-dependent activation of beta2-chimaerin restricts EGF-induced Rac signaling. *EMBO J.* 2006. **25:** 2062–2074.

46 **Leitges, M., Gimborn, K., Elis, W., Kalesnikoff, J., Hughes, M. R., Krystal, G. and Huber, M.,** Protein kinase C-delta is a negative regulator of antigen-induced mast cell degranulation. *Mol. Cell. Biol.* 2002. **22:** 3970–3980.

47 **Rusanescu, G., Gotoh, T., Tian, X. and Feig, L. A.,** Regulation of Ras signaling specificity by protein kinase C. *Mol. Cell. Biol.* 2001. **21:** 2650–2658.

48 **Parekh, A. B. and Penner, R.,** Depletion-activated calcium current is inhibited by protein kinase in RBL-2H3 cells. *Proc. Natl. Acad. Sci. USA* 1995. **92:** 7907–7911.

49 **Rambaratsingh, R. A., Stone, J. C., Blumberg, P. M. and Lorenzo, P. S.,** RasGRP1 represents a novel non-protein kinase C phorbol ester signaling pathway in mouse epidermal keratinocytes. *J. Biol. Chem.* 2003. **278:** 52792–52801.

50 **Yamashita, S., Mochizuki, N., Ohba, Y., Tobiume, M., Okada, Y., Sawa, H., Nagashima, K. and Matsuda, M.,** CalDAG-GEFIII activation of Ras, R-Ras, and Rap1. *J. Biol. Chem.* 2000. **275:** 25488–25493.

51 **Price, L. S., Langeslag, M., ten Klooster, J. P., Hordijk, P. L., Jalink, K. and Collard, J. G.,** Calcium signaling regulates translocation and activation of Rac. *J. Biol. Chem.* 2003. **278:** 39413–39421.

52 **Yang, F. C., Kapur, R., King, A. J., Tao, W., Kim, C., Borneo, J., Breese, R. *et al.*,** Rac2 stimulates Akt activation affecting BAD/Bcl-X_L expression while mediating survival and actin function in primary mast cells. *Immunity* 2000. **12:** 557–568.

53 **Melendez, A. J. and Khaw, A. K.,** Dichotomy of Ca^{2+} signals triggered by different phospholipid pathways in antigen stimulation of human mast cells. *J. Biol. Chem.* 2002. **277:** 17255–17262.

54 **Hanna, A. N., Berthiaume, L. G., Kikuchi, Y., Begg, D., Bourgoin, S. and Brindley, D. N.,** Tumor necrosis factor-alpha induces stress fiber formation through ceramide production: Role of sphingosine kinase. *Mol. Biol. Cell* 2001. **12:** 3618–3630.

55 **Hobson, J. P., Rosenfeldt, H. M., Barak, L. S., Olivera, A., Poulton, S., Caron, M. G., Milstien, S. and Spiegel, S.,** Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. *Science* 2001. **291:** 1800–1803.

56 **Biyasheva, A., Svitkina, T., Kunda, P., Baum, B. and Borisy, G.,** Cascade pathway of filopodia formation downstream of SCAR. *J. Cell Sci.* 2004. **117:** 837–848.

57 **McGough, A. M., Staiger, C. J., Min, J. K. and Simonetti, K. D.,** The gelsolin family of actin regulatory proteins: Modular structures, versatile functions. *FEBS Lett.* 2003. **552:** 75–81.

58 **Borovikov, Y. S., Norman, J. C., Price, L. S., Weeds, A. and Koffer, A.,** Secretion from permeabilised mast cells is enhanced by addition of gelsolin: Contrasting effects of endogenous gelsolin. *J. Cell Sci.* 1995. **108:** 657–666.

59 **Pollard, T. D. and Borisy, G. G.,** Cellular motility driven by assembly and disassembly of actin filaments. *Cell* 2003. **112:** 453–465.

60 **Barsumian, E. L., Isersky, C., Petrino, M. G. and Siraganian, R. P.,** IgE-induced histamine release from rat basophilic leukemia cell lines: Isolation of releasing and nonreleasing clones. *Eur. J. Immunol.* 1981. **11:** 317–323.

