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Review Article

Role of calcium, protein kinase C and MAP kinase in the activation of mast cells

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ABSTRACT

The mechanisms of activation of mast cells have been studied in most detail in rat RBL-2H3 cells. These cells respond to antigen via the IgE receptor (FcεRI) through sequential activation of the tyrosine kinases, Lyn and Syk, and to adenosine analogs via the adenosine A_3 receptor (A_3R) and a pertussis toxin-sensitive G protein, most likely G_{i-3}. Other receptors, introduced through gene transfection, include the muscarinic m1 receptor (m1R) which acts via $G_{q/11}$. Stimulation of cells via FcεRI, A_3R or m1R leads to the activation of phospholipase (PL) C, PLD and mitogen-activated protein (MAP) kinase resulting in the generation of inositol phosphates and diglycerides, an increase of cytosolic Ca^{2+}, the activation of protein kinase C (PKC) and the phosphorylation of various proteins by PKC and MAP kinase. The extent and time course of these events varies for each receptor. These variations, as well as the effects of pharmacologic probes, gene transfection and reconstitution of responses in washed permeabilized cells, indicate how these events relate to functional responses. A modest but sustained elevation of cytosolic Ca^{2+} through an influx of extracellular Ca^{2+} and activation of PKCβ and PKCδ are sufficient for optimal release of preformed secretory granules. Phosphorylation of a cytosolic PLA_2 by MAP kinase ($p42^{mapk}$) and a modest increase in cytosolic Ca^{2+} are necessary for the activation of PLA_2 and the binding of PLA_2 to membranes, respectively. Finally, both *de novo* generation and secretion via Golgi-derived vesicles of certain cytokines are dependent on Ca^{2+} and PKC as well as additional signals most probably phosphorylation of proteins by Syk and $p42^{mapk}$.

Key words: cytokines, lipid mediators, mast cells, secretion, signalling mechanisms

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INTRODUCTION

The role of mast cells and basophils in allergic disorders

High affinity receptors for immunoglobulin (Ig)E (FcεRI) are expressed exclusively on tissue mast cells and blood basophils. For this reason, these cells are primarily responsible for IgE-mediated allergic reactions. These cells are activated by multivalent binding of antigen to IgE that is bound to FcεRI, causing the rapid release of an array of potent inflammatory mediators by the discharge of the contents of secretory granules such as histamine, serotonin, proteases, proteoglycans and chemotactic peptides and by the activation of phospholipase A_2 with the release of arachidonic acid and its lipid metabolites, the prostanoids and leukotrienes. These cells also, after a delay of 30–60 min, generate and release cytokines such as tumor necrosis factor-α (TNF-α), the interleukins (IL) 1, 3, 4 and 6, and granulocyte-macrophage CSF.[1] Release of cytokines is sustained for a period of several hours.[2] These three types of response result in symptoms that are characteristic of immediate hypersensitivity reactions. Release of secretory granules and the arachidonic-acid-derived metabolites are thought to account for the familiar symptoms of hay fever, antigen-sensitive asthma, gastrointestinal hyper-sensitivity reactions and anaphylactic reactions to insect stings or injected proteins. There is now a substantial body of evidence that the release of cytokines, especially TNF-α, may be responsible, in part, for the delayed inflammatory responses in some of these reactions.[2] The signaling pathways for each of these three types of response and the effects of pharmacologic agents on these pathways is reviewed. Previously unpublished data, to assist our understanding of these pathways, is also presented.

In addition to FcεRI, mast cells also may express adenosine A_3 receptors,[3] IgG-binding receptors of the FcγRII and FcγRIII categories[4] and receptors for the complement-derived anaphylotoxins, C3a and C5a. These receptors may contribute further to mast-cell-mediated disorders.[5] FcεRI-mediated signals have been studied almost exclusively in the rat RBL-2H3 cell line, and

for this reason most of our discussion will focus on this cell line and its mutated forms.

RBL-2H3 cell as an experimental model for studying mast cell function

The RBL-2H3 cell has become a widely used surrogate for the study of antigen-induced responses in mast cells, partly because antigen responsive elements, namely IgE bound to FcεRI, can be experimentally manipulated in ways that are impossible with isolated tissue mast cells. The cells express, as do normal mast cells, several hundred thousand FcεRI and can be primed with monoclonal antigen-specific IgE or mixtures of IgE and myeloma IgE to vary the number of antigen-responsive elements.[6] They also express adenosine A_3 receptors,[3] and FcγRIII,[4] and, for comparative purposes, have been made to express various G-protein-coupled receptors by gene transfection (see below). Of these receptors, FcεRI, adenosine A_3 and transfected muscarinic m1 receptors have been studied in most detail. In addition, this cell line exhibits the same functional responses to antigen as normal mast cells. As these responses are readily measured in either 24- or 96-well culture plates, RBL-2H3 cells can be used for studying signal transduction mechanisms in general.

The phenotype of the RBL-2H3 cell is still a matter of debate but on the basis of biochemical criteria[7] they resemble rat mucosal mast cells more than the connective tissue mast cells. Also, like the mucosal mast cell, RBL-2H3 cells do not respond to polybasic compounds such as compound 48/80 and polybasic neuropeptides.[8] Changes, reminiscent of a shift in phenotype, are induced by co-culture with 3T3 fibroblasts. During co-culture, RBL-2H3 cells become responsive to the polybasic secretagogs.[9]

Source of monoclonal IgE and experimental protocols for the activation of RBL-2H3 cells

The discovery of rat immunocytomas that secreted IgE10 permitted the preparation of antigen-specific mouse IgE in high yields. One of these (HI-DNP-ε-26.82) has been widely used11 for sensitizing RBL cells to the antigens dinitrophenylated bovine serum albumin (DNP-BSA) and horse serum albumin (DNP-HSA). Typically, RBL-2H3 cells are incubated with the monoclonal DNP-specific IgE overnight in complete growth medium. Radiolabeled inositol, serotonin, arachidonic acid or other radiolabeled metabolites may be included to label metabolic pools. The next day, the medium is replaced with a simple buffered salt-glucose medium. The cultures are then stimulated by the addition of DNP-BSA or any other stimulant for measuring the release of granule constituents (e.g. histamine, [3H]serotonin or hexosaminidase)[12,13] or radiolabeled metabolites such as inositol phosphates,14 arachidonic acid,15 phosphatidic acid and various phospholipids16 or unlabeled products such as cytokines and diglycerides by ELISA or enzymatic assay procedures.[13,16]

SIGNALS GENERATED THROUGH FcεRI AND OTHER RECEPTORS

Initial signaling events via FcεRI

FcεRI is a member of the family of multimeric immunoglobulin-binding receptors which, in common with the T-cell- and B-cell-antigen receptors, recruit cytosolic tyrosine kinases for the initiation of stimulatory signals.[1,17] FcεRI consists of the IgE-binding α subunit, a β subunit and a disulfide-linked homologous dimer of γ chains.[6] The binding of multivalent antigen to receptor-bound IgE induces an aggregation of FcεRI and this aggregation is the trigger for cell activation. Activation can be achieved by direct cross-linking of receptors with covalent oligomers of the Fc fragments of myeloma-IgE protein.[6]

Recent studies with RBL-2H3 cells provide a scenario as to how the aggregation of receptors generate biochemical signals within the cell. The cytosolic domains of the β and γ subunits of FcεRI, like the ζ chain of the T cell antigen receptor, contain sequence motifs (ITAM, immunoreceptor tyrosine-based activation motif)[18] which, when phosphorylated, tag SH2-domains of cytosolic tyrosine kinases and other signal-transducing proteins such as Shc (see section on Activation of MAP kinase). In fact, the cytosolic portions of the FcεRI γ chain and the ζ chain of the T cell receptor are interchangeable with little detriment to signal transduction.[17,19] Expression of TAC-chimeric constructs of the β and γ chains of FcεRI[19] and biochemical studies[20,21] suggest the following sequence of events. The tyrosine kinase, Lyn ($p56^{lyn}$), is normally associated with the β chain in a constitutively active form. Aggregation of FcεRI, by bringing Lyn into close proximity to ITAM of the β and γ chains of adjacent receptors, allows tyrosine phosphorylation of these sites. This transphosphorylation promotes the additional recruitment of Lyn by the β chain, the recruitment of another tyrosine kinase, Syk ($p72^{syk}$), by the γ chain and the resultant tyrosine phosphorylation of other proteins by Syk.[19] The tyrosine phosphorylation of the phospholipase Cγ1[22] and γ2 (OH Choi *et al.*, 1944, unpubl. data), and the apparent activation of these isozymes[22] in RBL-2H3 cells is most probably mediated by Syk although this has not been unequivocally proven. It has been established, however, that Syk is responsible for the tyrosine phosphorylation of various proteins, secretion of granules[19] and activation of the MAP kinase/phospholipase A_2 cascade of signals[23] as these events are blocked in RBL-2H3 cells by the introduction of a gene for truncated Syk which lacks the kinase domain (see later sections).

Adenosine receptors: Finding a novel receptor coupled to phospholipases C and D

The adenosine receptors on RBL-2H3 cells, like those on rat peritoneal cells, are capable of synergizing antigen-induced signals for secretion but by themselves promote little secretion when stimulated with adenosine analogs. These receptors differ from classic adenosine A_1 and A_2 receptors in their inability to

alter levels of cyclic AMP[24] and their resistance to xanthine antagonists of the A_1 and A_2 receptors.[25] RBL-2H3 cells and other mast cell lines express high levels of mRNA transcripts of the A_3 receptor[3] and low levels of mRNA transcripts for the A_{2a} and A_{2b} receptors.[26] The evidence to date suggests that adenosine-induced responses in RBL-2H3 cells are mediated predominantly, if not exclusively, through the A_3 receptor.[27]

Stimulation via the A_3 receptors causes transient activation of phospholipase C[24] and sustained activation of phospholipase D and protein kinase C[27] by a process that is inhibited by both cholera and pertussis toxins[24] and markedly enhanced by treatment with dexamethasone such that cells now secrete in response to adenosine analogs.[28,29] As dexamethasone increases the expression of the A_3 receptor as well as the α and β subunits of several trimeric G proteins,[30] it has been suggested that both the α and $\beta\gamma$ subunits of G proteins contribute to the activation of these two phospholipases.[27] The sustained activation of phospholipase D and protein kinase C probably accounts for the ability of adenosine analogs to synergize secretory responses to antigen and other secretagogs.[27] Activation of phospholipase D results in the formation of phosphatidic acid and, via phosphatidate hydrolase, diglycerides which, in turn, activate protein kinase C (see below).

Expression of other types of receptors by gene transfection

Because the repertoire of receptors is limited, the utility of the RBL-2H3 cell line has been enhanced by the expression of other receptors by gene transfection. The cell line has been stably transfected with genes for the muscarinic m1[31] and m3[32] receptors. Both sublines respond to carbachol by the activation of phospholipase C-mediated signals and secretion indicating that RBL-2H3 cells contain appropriate coupling-proteins for these receptors. The muscarinic m1 receptors are coupled to phospholipase Cβ3 through $G_{\alpha q/11}$, as indicated by the selective downregulation of these proteins after prolonged exposure of the cells to the m1 agonist, carbachol, but not after exposure to antigen. Carbachol does not induce tyrosine phosphorylation of phospholipase C γ1 and γ2 as does antigen (Choi OH, Yamada K & Beaven MA, 1994, unpubl. obs.), but both stimulants elicit a similar array of responses.[31,33] As will be elaborated upon later, the notable differences are that carbachol is a much weaker stimulant of TNF synthesis than antigen[34] and that the two stimulants activate the MAP kinase pathway by different mechanisms.[23]

The anaphylatoxins, C3a and C5a, are potent stimulants of mast cells and basophils, respectively.[5] RBL-2H3 cells possess no detectable receptors for C5a or other chemotactic peptides.[35] Sublines expressing epitope-tagged receptors for the chemotactic peptide, fMLP,[35] C5a[35,36] as well as platelet activating factor,[37] IL-8[38] or thrombin[39] have been produced for studies of receptor desensitization. The studies indicate that heterologous desensitization is associated with phosphorylation of some of these receptors by protein kinase C whereas homologous desensitization is attributable to phosphorylation of all of these receptors through phosphorylation by G-protein-coupled receptor kinases (GRK).[38,40] All of these sublines can be stimulated via these expressed receptors to elicit the full phospholipase C-mediated cascade of signals including the release of secretory granules to further indicate that RBL-2H3 cells possess the mechanisms for coupling and regulating both G protein and tyrosine kinase dependent receptors.

Chemical stimulants of RBL-2H3 cells

Stimulants that bypass early receptor-mediated stimulatory events include the Ca^{2+}-ionophores, ionomycin and A23187, and activators of protein kinase C such as phorbol 12-myristate 13-acetate. Low concentrations of these reagents (<100 nmol/L) elicit respectively, substantial elevation of $[Ca^{2+}]_i$ and activation of protein kinase C but they do not induce the release of secretory granules. In combination, however, they stimulate secretion.[41–43] Thapsigargin, which elevates $[Ca^{2+}]_i$ by blocking the uptake of Ca^{2+} into IP_3-sensitive stores, also stimulates secretion but only at concentrations (>100 nmol/L) far in excess of those (10 nmol/L) required for the elevation of $[Ca^{2+}]_i$.[44] While these studies suggest that an increase in $[Ca^{2+}]_i$ and in protein kinase C provide signals for secretion, it should be noted that high concentrations of Ca^{2+}-ionophore stimulate phospholipase C[43] and phospholipase D.[45]

Intermediate signaling events in RBL-2H3 cells and mutated sublines

Recruitment of phospholipases C and D

RBL-2H3 cells possess the β2, β3, γ1 and γ2 forms of phospholipase C (Hirasawa N & Beaven MA, unpubl. data) as well as phospholipase D activity.[46] The activation of the β isoforms through G-protein-coupled receptors and the tyrosine phosphorylation of the γ-isoforms[22] of phospholipase C through activation of Lyn/Syk tyrosine kinases via FcεRI[1,19,47] has been noted earlier. The mechanisms of activation of phospholipase D are still undetermined, but the recent cloning of a gene that encodes one form of phospholipase D should facilitate studies of these mechanisms.[48]

Mobilization of intracellular and extracellular calcium ions

Early studies established that degranulation of rat peritoneal mast cells is dependent on external Ca^{2+} and is associated with influx of Ca^{2+} ($^{45}Ca^{2+}$) and other divalent cations.[49,50] This influx is associated with the generation of second messengers and is reminiscent of what has been observed in other types of electrically non-excitable cells[33] in which there is rapid release of Ca^{2+} from inositol 1,4,5-trisphosphate-sensitive Ca^{2+}-stores followed by an influx of Ca^{2+}.[51,52] This influx is closely associated with the emptying of inositol 1,4,5-trisphosphate-sensitive Ca^{2+}-

stores[51,52] and the generation of a diffusible messenger molecule[53,54] from intracellular organelles. Influx is thought to occur through a low conductance current, designated I_{crac} for 'calcium release-activated calcium current', which has been characterized in mast cells[55] and RBL-2H3 cells.[56] This current appears to be highly selective for Ca^{2+} ions.[55]

In the RBL-2H3 cell, antigen stimulation causes, after a short delay, a transient increase in $[Ca^{2+}]_i$ in the absence of external Ca^{2+} [31,57,58] which has been attributed to the release of Ca^{2+} from intracellular stores by inositol 1,4,5-trisphosphate.[31,33] Stimulation in the presence of external calcium results in a sustained increase in $[Ca^{2+}]_i$. The sustained elevation in $[Ca^{2+}]_i$, as determined by Ca^{2+}-sensitive fluorescent probes, is totally dependent on the influx of external Ca^{2+}.[59] The increase in $[Ca^{2+}]_i$ is associated with an increase in total intracellular Ca^{2+} possibly due to the uptake of cytosolic Ca^{2+} into mitochondrial stores when $[Ca^{2+}]_i$ is elevated above basal levels.[44,60] Other cations impede Ca^{2+}-uptake, either by blocking the entry of Ca^{2+} at the cell surface or by competing for Ca^{2+}-entry into the cell.[50] Entry of Ca^{2+} is suppressed also when cells are depolarized by high concentrations of external K^+ [61,62] and a repolarizing current may be required to maintain influx.[62-67] In addition to I_{crac}, novel sphingolipid-gated Ca^{2+}-gated efflux channel (from Ca^{2+}-storage organelles) has recently been described in RBL-2H3 cells[68] but its physiological relevance remains undetermined.

Recruitment of protein kinase C: Actions of individual isozyme agonists and inhibitors

Protein kinase C is a family of serine/threonine kinases that are rapidly activated in response to elevated $[Ca^{2+}]_i$ and the generation of diglycerides via phospholipases C and D. The isoforms differ in their requirements for calcium and lipid co-factors which allows activation of the enzyme in various microenvironments. Phorbol esters can substitute for diacylglycerol in activating protein kinase C and have been widely used in unmasking protein-kinase-C-mediated phosphorylations and actions *in vivo*. The isoforms have been categorized as conventional or calcium- dependent (α, the alternatively spliced variants βI and βII, and γ), novel or calcium-independent (δ, ϵ, π, ϕ, and μ), and atypical (ζ and λ) on the basis of their diverse properties and their historical sequence of discovery. The atypical are the least understood category of isoforms but they fail to respond to phorbol esters.[69] Distinct structural/topographical differences among these categories account for the diverse properties and, it is believed, permit the individual isoforms to subserve different functions within the cell.[70,71] Indeed, this is strongly supported by studies in RBL-2H3 cells.

RBL-2H3 cells contain the Ca^{2+}-dependent α, βI and βII isoforms and the Ca^{2+}-independent δ, ϵ and ζ isoforms of protein kinase C.[72-74] When cells are stimulated with antigen, these isoforms rapidly associate to variable extents (i.e. δ the most and ζ the least) with the membrane fraction but without external Ca^{2+}, only the Ca^{2+}-independent isoforms do so.[72] Washed permeabilized cells lose all isozymes of protein kinase C, and do not secrete in response to antigen and carbachol,[72] but stimulatory signals such as hydrolysis of inositol phospholipids are enhanced by as much as two- to three-fold.[75]

Reconstitution of antigen-induced responses by provision of recombinant isozymes of protein kinase C to washed permeabilized cells have suggested that antigen-induced secretion of granules is mediated primarily by protein kinase Cβ and δ[72] and feedback inhibition of phospholipase C is mediated primarily by protein kinase Cα and ϵ.[75] Similar studies have indicated that in antigen-stimulated cells, protein kinase Cβ and ε transduce signals for the expression of the c-*fos* and c-*jun*[76] and that protein kinase Cδ, by phosphorylating the γ subunit of FcϵRI specifically, may promote endocytosis of the receptor.[73] The isoforms also exhibit different rates of degradation when RBL-2H3 cells are continuously exposed to phorbol 12-myristate 13-acetate. Protein kinase Cβ and α are degraded within minutes and hours respectively, whereas the Ca^{2+}-independent isozymes (δ, ϵ and ζ) resist degradation.[72,77,78]

In addition to reconstitution studies with permeabilized cells, the role of protein kinase C in cell function has been studied typically by using phorbol esters to activate or selectively degrade isoforms of protein kinase C and of inhibitors of protein kinase C. It has been our experience that kinase inhibitors, in general, rarely have the selectivity intended when used *in vivo*.[79-81] Of the many drugs that we have tested, only the Ro series of protein kinase C inhibitors exhibit such selectivity.[82] One of them, Ro31-7549, suppresses the release of secretory granules and TNF without affecting activation of tyrosine kinases, myosin light chain kinase, and the MAP kinase/PLA_2 pathway.[34,80,81,83]

The activation of MAP kinase

Tyrosine kinase-dependent receptors, such as the EGF receptor and the multimeric immune receptors, utilize the SH2-containing protein Shc, the adaptor protein Grb2, and the guanine nucleotide exchange factor Sos, to convert Ras (p21*ras*) to its active GTP-bound state[84-86] which, in turn activates the MAP kinase pathway via Raf1.[87] Activation is accomplished through Ras-mediated translocation of the kinase Raf1, which when phosphorylated by an unidentified kinase, phosphorylates and activates a unique tyrosine/threonine kinase, MEK, which then phosphorylates and activates MAP kinase.[88]

The cascade of events has been demonstrated in antigen-stimulated RBL-2H3 cells as indicated by the interactions of Shc and Sos with Grb2, the activation of Ras, and the phosphorylation of Shc,[89] Raf1, MEK and $p42^{mapk}$.[83,90] The tyrosine phosphorylation of $p42^{mapk}$ is associated with a shift in electrophoretic migration of $p42^{mapk}$ [90] and an increase in MAP kinase activity.[23,83,91] Expression of porcine Syk, or a dominant-negative truncated Syk (Syk-T) that lacks the kinase domain,[23,92] in a vaccinia expression system, has indicated that Syk is essential for activation of the Shc/Grb2/Sos[89] and MAP kinase[23] pathways when cells are stimulated by antigen.

The MAP kinase pathway may be activated in RBL-2H3 cells

through alternate pathways.[83] Activation of the Raf/Mek/MAP kinase cascade via the muscarinic m1 receptors, for example, is not dependent on Syk.[23] Current studies suggest that elevated $[Ca^{2+}]_i$ and protein kinase C may provide alternate signals for activation (C Zhang, N Hirasawa & MA Beaven, unpubl. data). Also, it is unclear whether Syk-dependent tyrosine phosphorylation of Vav in antigen-stimulated RBL-2H3 cells provides yet another mechanism of activation.[23]

Late signaling events

The targets for signals transduced via calcium and protein kinase C that ensure activation of the secretory machinery in RBL-2H3 cells have not been identified, but potential targets are the light and heavy chains of myosin. In stimulated RBL-2H3 cells, myosin light and heavy chains are phosphorylated by protein kinase C[93] and the light chains by both protein kinase C and myosin light chain kinase.[81,94] These phosphorylations show close correlation with the rate and the extent of degranulation when cells are stimulated with antigen and chemical secretagogs.[81,93] Although these studies and those with inhibitors of protein kinase C and calcium, demonstrate a close correlation between phosphorylation and degranulation, they do not establish a causal relationship. RBL-2H3 cells express only one (myosin-A) of the two (myosin-A and B) isoforms of myosin,[95] and studies with antibodies against myosin-A in permeabilized cells should be instructive.

Another area of current interest is the role of G proteins regulating granule trafficking and fusion. This interest stemmed from the observation of Gomperts and co-workers that Ca^{2+} and non-hydrolyzable GTP analogs together were sufficient to induce maximal secretory response in permeabilized[96] and patch-clamped mast cells.[97] Because elevation in $[Ca^{2+}]_i$ eliminated the requirement for the GTP analog, and vice versa, it was hypothesized that a Ca^{2+}-receptor (called C_e, where e stands for exocytosis) required a G protein (called G_e) for further transduction of signals. There is evidence that a pertussis toxin-sensitive, trimeric G protein,[98] most likely G_{i13}, mediates a late step in exocytosis and is directly activated by compound 48/80 in rat peritoneal mast cells.[99] Other studies in which exocytosis was induced in rat mast cells by microinjection of the constitutively activated product of the H-*ras* oncogene[100] or peptide analogs of Rab3a[101] suggst that low molecular weight monomeric G proteins may also serve the function of G_e.

SIGNALING EVENTS FOR RELEASE OF SECRETORY GRANULES, ARACHIDONIC ACID, AND CYTOKINES

Activation of protein kinase C and elevation of $[Ca^{2+}]_i$ are necessary and sufficient signals for secretion

The studies with pharmacologic stimulants (see section on Chemical stimulants of RBL-2H3 cells) suggest that elevation of $[Ca^{2+}]_i$ and activation of protein kinase C provide signals for secretion in RBL-2H3 cells, and the pattern of phosphorylation of myosin chains implies that these two signals are active in antigen-stimulated cells. Also, blockade of secretion by either Ro31-7549 or the calcium chelator, EGTA, establishes that these are two necessary signals for secretion.[72] However, these findings do not prove that these are the only physiologic signals for secretion.[42,102]

The most definitive information on the role of calcium and protein kinase C in secretion has come from reconstitution studies in permeabilized RBL-2H3 cells. As previously noted, washed permeabilized cells lose all isozymes of protein kinase C and fail to secrete in response to antigen. A full secretory response to antigen could be reconstituted by the subsequent addition of nmol/L concentrations of either the Ca^{2+}-dependent protein kinase Cβ or the Ca^{2+}-independent protein kinase Cδ (other isozymes were much less effective), but only in the presence of 1 μmol/L free Ca^{2+} to indicate separate roles for Ca^{2+} and protein kinase C in exocytosis.[72]

To demonstrate that signals generated via calcium and protein kinase C provide sufficient signals for secretion, secretion was induced in washed, permeabilized cells by the addition of the protein kinase C agonist, 1-oleoyl 2-acetyl-sn-glycerol (OAG), and protein kinase Cδ, which does not require calcium for activation in the presence of various $[Ca^{2+}]_i$. Control experiments indicated that the elevation of $[Ca^{2+}]_i$ only did not stimulate secretion even when $[Ca^{2+}]_i$ was raised to 10 μmol/L, although these high, non-physiologic concentrations did stimulate a slight release of inositol phosphates and arachidonic acid (Fig. 1). In the presence of protein kinase Cδ, physiologic concentrations of calcium (0.1 μmol/L and 1 μmol/L) induced no or little (>3%) secretion, but high $[Ca^{2+}]_i$ induced moderate secretion possibly as a consequence of the stimulation of lipid metabolism (Fig. 2a). In the presence of 10 μmol/L OAG and protein kinase Cδ, a small elevation of $[Ca^{2+}]_i$ elicited a secretory response similar to that in antigen-stimulated cells (approximately 40%). The data indicated a half maximal response (EC_{50}) at about 200 nmol/L $[Ca^{2+}]_i$, and near maximal response at 700–1000 nmol/L $[Ca^{2+}]_i$ (Fig. 2b), responses that were comparable to those observed in intact antigen-stimulated cells.[59,103]

Further definition of the roles of calcium and protein kinase C in secretion in antigen-stimulated RBL-2H3 cells

Protein kinase Cδ is known to translocate to the membrane in response to antigen stimulation in the absence of external calcium,[72] but as noted earlier, the calcium-dependent protein kinase Cβ also transduces a signal for secretion in permeabilized cells and its translocation to the membrane is dependent on calcium.[72] As protein kinase Cβ is the most potent of the two isoforms in promoting secretion,[72] there are at least two requirements for calcium in intact cells; one, as an activator of protein

kinase Cβ, the other as a signal for secretion. These requirements were examined in two additional experiments with washed permeabilized cells as described below.

In the first experiment, $[Ca^{2+}]_I$ was varied from 10 nmol/L to 1000 nmol/L in the presence of 100 nmol/L protein kinase Cδ, or 10 nmol/L protein kinase Cβ. These concentrations were known to sustain maximal responses to antigen.[72] The ability of these cells to secrete in response to antigen indicated that the requirement for $[Ca^{2+}]_I$ was the same for either isoform (Fig. 3a). Fifty percent of the maximal secretory response to antigen (EC_{50}) was observed with 190–210 nmol/L $[Ca^{2+}]_I$, and near maximal response, with 400–600 nmol/L $[Ca^{2+}]_I$ (Fig. 3a). These values corresponded closely to those obtained in the studies with OAG (Fig. 2).

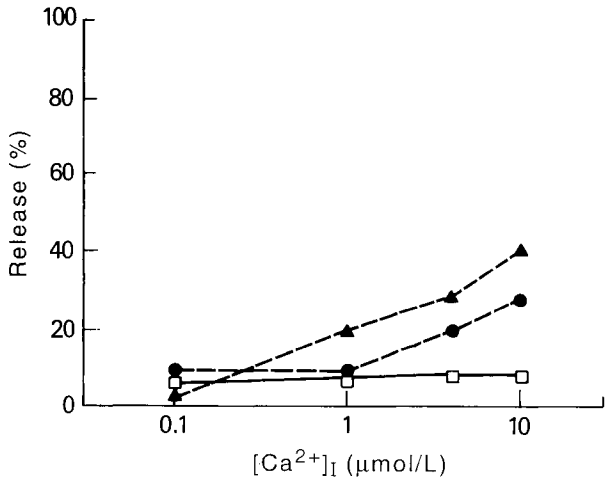

Fig. 1 Stimulation of release of arachidonic acid (▲), inositol phosphates (●), but not of hexosaminidase (□), a granule marker, in washed permeabilized RBL-2H3 cells at various concentrations of buffered calcium. Values indicate percent release at 15 min[72, 75,83] and are expressed as mean ± SEM of three cultures.

The second experiment was designed to assess the requirement for $[Ca^{2+}]_I$ for the activation of protein kinase Cβ (Fig. 3b). The experiment was performed as described above except that the cells were washed shortly after the addition of antigen to remove excess protein kinase C that had not translocated to the membrane. The medium was then replaced with medium that contained 1000 nmol/L $[Ca^{2+}]$ to ensure complete release of secretory granules. The extent of secretion was assumed to be dependent on the amount of isozyme retained and activated within the cell. The activation of protein kinase Cβ, but not of protein kinase Cδ, appeared to be highly dependent on the initial concentration of calcium (Fig. 3b). The leftward shift in the curve for protein kinase Cβ indicated that the requirement for $[Ca^{2+}]_I$ for activation of protein kinase Cβ was less than that for secretion (Fig. 3a,b). Half-maximal (EC_{50}) and near maximal responses were achieved with 110 nmol/L and 250 nmol/L $[Ca^{2+}]_I$, respectively. Thus, relatively small increases $[Ca^{2+}]_I$ were required for activation of the β isoform.

Further studies revealed that permeabilized RBL-2H3 cells exhibited quantal release of granules when the concentration of protein kinase C was limited (Fig. 4a,b). In this series of experiments, washed permeabilized cells were exposed to 10 nmol/L $[Ca^{2+}]_I$ and to different concentrations of protein kinase Cβ or δ before the addition of antigen. Secretion was then initiated by raising $[Ca^{2+}]_I$ to 1 μmol/L. Secretion was essentially complete within 10 min in the presence of 10 nmol/L protein kinase Cβ

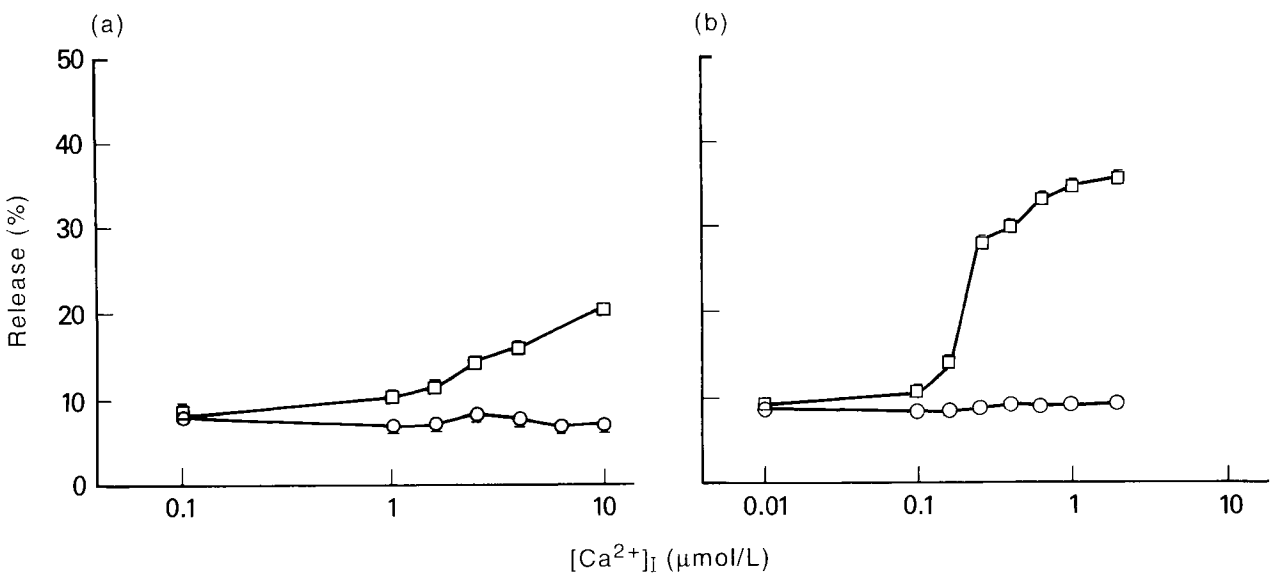

Fig. 2 Stimulation of release of hexosaminidase in washed permeabilized cells with (□) or without (○) 100 nmol/L protein kinase Cδ (a) in the absence or (b) in the presence of 10 μmol/L 1-oleoyl 2-acetyl-sn-glycerol (OAG) at various concentrations of buffered calcium. Values indicate percent release at 15 min and are expressed as mean ± SEM of three cultures.

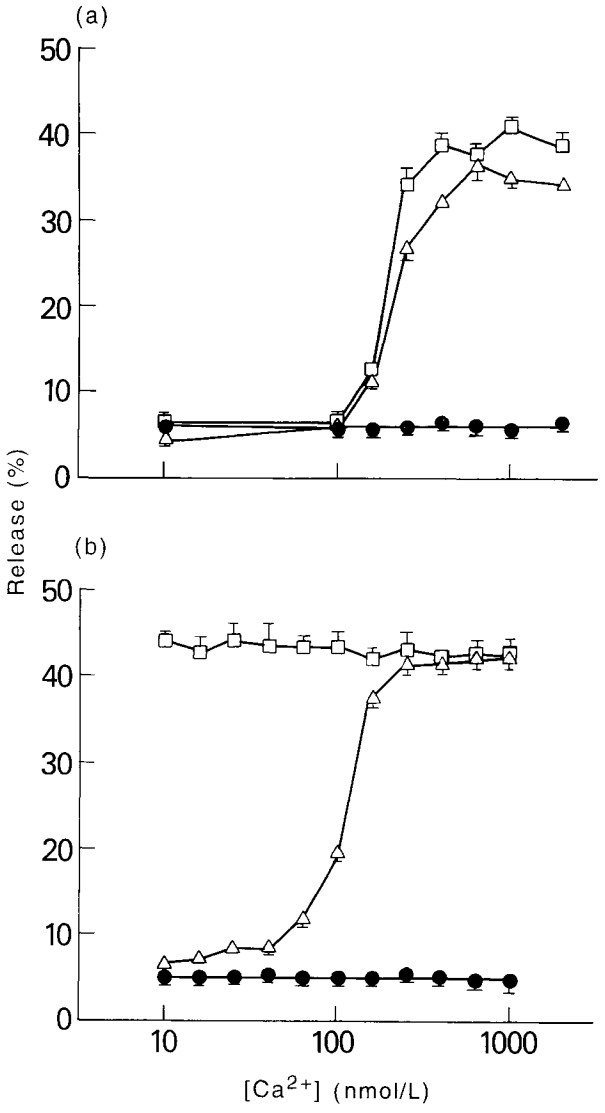

Fig. 3 (a) Calcium requirement for antigen-stimulated release of hexosaminidase by washed permeabilized cells incubated (15 min) with 100 nmol/L protein kinase Cδ (□), 10 nmol/L protein kinase Cβ (△), or no protein kinase (●). (b) Calcium requirement for activation of protein kinase C in permeabilized cells incubated with protein kinase Cβ (△), protein kinase Cδ (□) and without protein kinase (●). Calcium was then increased to 1 μmol/L to ensure complete release. Values are expressed as mean ± SEM of three cultures.

(Fig. 4a) and within 1 or 2 min in the presence of 100 nmol/L protein kinase Cδ (Fig. 4b). With less than optimal concentrations of either isozyme, the extent but not the time course of secretion was altered (Fig 4a,b). These kinetics implied that the cells responded quantally to protein kinase C and that each molecule of membrane-associated protein kinase C had access to limiting amounts of substrate that was critical for secretion.

The studies mentioned previously also demonstrated that the secretory machinery in RBL-2H3 cells responded relatively rapidly to stimulatory signals. Equally rapid responses could be invoked in intact cells when the addition of calcium was delayed until after the addition of antigen (Fig. 4c). As in other studies,[59,93] the time course of secretion when intact cells were stimulated in the presence of calcium was relatively slow (Fig. 4d). The difference in time course suggested that the rate-limiting step in secretion in the intact cell was the initiation of stimulatory signals that preceded calcium mobilization in response to antigen-stimulation.

In summary, these studies have indicated that an increase in $[Ca^{2+}]_i$ and activation of protein kinase Cβ or δ provide sufficient signals for secretion. Relatively modest increases in $[Ca^{2+}]_i$ are necessary for mediating secretion and even smaller increases are required for translocation and activation of protein kinase Cβ but not the δ isoform. The quantal response of cells to both isoforms of protein kinase C is intriguing and warrants further investigation.

Synthesis and release of arachidonic acid

The effector enzyme for receptor-mediated release of arachidonic acid in various cells[104,105] is now thought to be the high molecular weight PLA_2, usually referred to as cytosolic PLA_2 ($cPLA_2$), that has cDNA that has been cloned from human monoblast[105–107] and murine macrophage[106] cell lines. The deduced amino-acid sequence (85 kDa) indicates exceptional similarity between the human and murine forms of the enzyme, a shared Ca^{2+}-dependent phospholipid binding domain and a single serine-containing consensus site for phosphorylation by MAP kinase.[106] These and other findings suggest that $cPLA_2$ activity is regulated primarily by calcium and by serine phosphorylation of $cPLA_2$ by MAP kinase.[104,105] As noted earlier, activation of this pathway is achieved by sequential activation of Ras, Raf1, MEK1, and MAP kinases.[88,108] Activation of this cascade of reactions is thought to result in the activation of $cPLA_2$ and there is evidence that this might be the case in RBL-2H3 cells.

Stimulation of intact or permeabilized RBL-2H3(m1) cells with antigen, carbachol, A23187 or thapsigargin results in the apparent activation of Raf1, MEK1, MAP kinase, $cPLA_2$ as well as the release of arachidonic acid.[83] The entire pathway is inhibited by low concentrations of quercetin, but not by Ro31-7549, and thus appears to be dependent on a quercetin-sensitive protein kinase that is not protein kinase C.[83] These and other findings indicate that release of arachidonic acid is attributable exclusively to the regulation of $cPLA_2$ by MAP kinase (for activation of $cPLA_2$) and Ca^{2+} (for association of $cPLA_2$ with the membrane).[23]

The over-expression of Syk or truncated SykT in RBL-2H3(m1) cells (see section on The activation of MAP kinase) indicated that antigen-induced activation of $cPLA_2$ and the release of arachidonic acid, as well as the activation of MAP kinase, were dependent on Syk. The role of Syk in mediating signals via FcεRI was also evident in RBL-2H3 cells that expressed the TAC chimeras of the β and γ chains of FcεR. These cells respond to cross linking of TACγ with

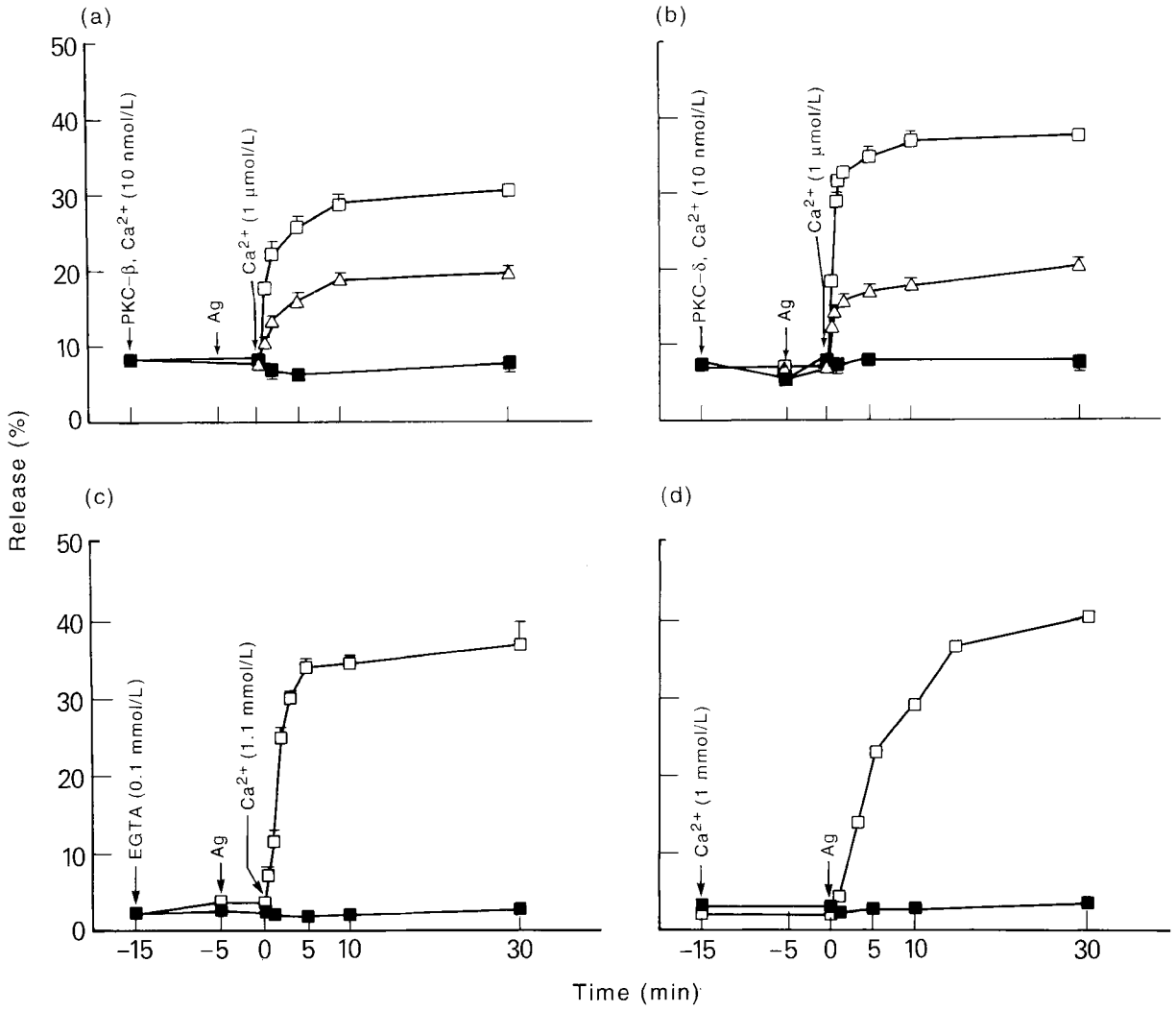

Fig. 4 Time course of antigen (Ag; 10 ng/mL DNP-BSA) stimulated secretion in (a, b) washed, permeabilized and (c,d) intact RBL-2H3 cells. The additions (indicated by arrows) in: (a) 5 nmol/L (△) or 10 nmol/L (□,■) protein kinase Cβ, 10 nmol/L (■) or 1 µmol/L (□,△) free calcium; (b) 30 nmol/L (△) or 100 nmol/L (□,■) protein kinase Cδ, 10 nmol/L (■) or 1 µmol/L (□,△) free calcium; (c) 0.1 mmol/L EGTA followed by no (■) or 1.1 mmol/L calcium and; (d) no (■) or 1 mmol/L (□) calcium. Methods are as described elsewhere.[72] Values indicate percentage release of hexosaminidase and are the mean ± SEM of three cultures.

the activation of MAP kinase and $cPLA_2$ along with release of arachidonic acid. Again these responses are blocked by overexpression of SykT. As these same events could be induced by carbachol when Syk was inactivated by SykT, alternate pathways must exist for the activation of $cPLA_2$ via Ras and MAP kinase.[23]

Synthesis and release of cytokines

TNF-α is associated with discharged granules in human[109] and rodent[110–112] mast cells, and is presumably released by exocytotic discharge of these granules. In most cultured mast cell lines, however, cytokines are not constitutively expressed and in all types of mast cells there is increased expression of the cytokine protein or its mRNA, which is detectable from 30 min to several hours after the addition of a stimulant. Stimulation of cytokine production via FcεR1 is probably dependent on the mobilization of Ca^{2+} and activation of protein kinase C, because such production can be induced by calcium ionophore or the protein kinase C-activator, phorbol 12-myristate 13-acetate.[110,113,114] It has been postulated, however, that FcεR1-activated kinases may have a more direct role in stimulating cytokine production through the tyrosine phosphorylation of other substrates.[17] It has been argued that these early events result in either increased expression of cytokine mRNA by activation of gene transcription[17,115] or stabilization of short-lived mRNA transcripts[116] by Ca^{2+}-dependent kinases.[117]

Studies in RBL-2H3 cells[34] indicate that TNF is not constitutively expressed nor incorporated into secretory granules but is

generated *de novo* upon cell stimulation. Production of TNF is dependent on elevation of $[Ca^{2+}]_i$ and protein kinase C. Optimal production of TNF, however, may be dependent on additional synergistic signals as carbachol, which does not operate through Syk, is a weak stimulant of TNF production. Therefore, a Syk-dependent pathway may provide such synergistic signals. TNF is released from cells by a process analogous to constitutive secretion in that brefeldin A, an agent known to disrupt Golgi membranes in these cells, inhibits this release without inhibiting the release of secretory granules. Unlike constitutive secretion, the secretion of TNF is highly regulated by Ca^{2+} and protein kinase C. Studies with various stimulants and inhibitors have indicated that simultaneous mobilization of Ca^{2+} and activation of protein kinase C are sufficient signals for secretion and are potential targets for therapeutic intervention.

The role of MAP kinase is currently under investigation in our laboratory because over-expression of genes for Raf1[118] or MAP kinase[119] enhance the expression of a variety of cytokine genes in T cells and macrophages, the inactivation of IκB[120] and enhanced binding activity of cytokine transcription factors, including NF-κB and AP1.[119]

Future directions

We have presented evidence, based mostly on studies in RBL-2H3 cells, that the secretion of granules is regulated by protein kinase C and that the release of arachidonic acid, via PLA_2, is regulated by MAP kinase and that both processes are dependent on modest increases in $[Ca^{2+}]_i$. The communicating links between FcεR1 and these two kinases have been established, at least in broad detail. The remaining challenges are the identification of the events after the activation of protein kinase C and increased $[Ca^{2+}]_i$ for secretion and the the events related to the regulation of transcription factors for the cytokine genes, in particular the role of protein kinase C, MAP kinase and calcium. We suspect that the regulation of cytokine secretion via Golgi might also be a rewarding area of research.

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