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 A1 receptor (A1R) and a pertussis toxin-sensitive G protein, most likely G_i. Other receptors, introduced through gene transfection, include the muscarinic m1 receptor (m1R) which acts via G_q. Stimulation of cells via FcεRI, A1R 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 PLA2 by MAP kinase (p42mapk) and a modest increase in cytosolic Ca^{2+} are necessary for the activation of PLA2 and the binding of PLA2 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 p42mapk.

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

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 A2 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. Release of cytokines is sustained for a period of several hours. 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. 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 A2 receptors, IgG-binding receptors of the FcγRII and FcγRIII categories and receptors for the complement-derived anaphylatoxins, C3α and C5α. These receptors may contribute further to mast-cell mediated disorders. FcεRI-mediated signals have been studied almost exclusively in the rat RBL-2H3 cell line, and
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. They also express adenosine A2 receptors, and FcεRIII, and, for comparative purposes, have been made to express various G-protein-coupled receptors by gene transfection (see below). Of these receptors, FcεRI, adenosine A2, 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 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. 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 compounds.

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 dinitrophenolated bovine serum albumin (DNP-BSA) and horseradish peroxidase. 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, 1,4 arachidonic acid, 1,5 phosphatidic acid and various phospholipid16 or unlabeled products such as cytokines and diglycerides by ELISA or enzymatic assay procedures.14,15

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.1,14 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 ε 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 ε chain and the ε 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 FcεRI19 and biochemical studies20,21 suggest the following sequence of events. The tyrosine kinase, Lyn (p56<sup> lyn </sup>), 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<sup> syk </sup>), by the γ chain and the resultant tyrosine phosphorylation of other proteins by Syk.18 The tyrosine phosphorylation of the phospholipase Cy1 and y2 (OH Choi et al., 1944, unpubl. data), and the apparent activation of these isozymes 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 granules18 and activation of the MAP kinase/phospholipase A<sub>2 </sub>cascade of signals22 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<sub>1 </sub>and A<sub>2 </sub>receptors in their inability to...
alter levels of cyclic AMP and their resistance to xanthine antagonists of the A1 and A2 receptors. RBL-2H3 cells and other mast cell lines express high levels of mRNA transcripts of the A1 receptor and low levels of mRNA transcripts for the A2a and A2b receptors. The evidence to date suggests that adenine-induced responses in RBL-2H3 cells are mediated predominantly, if not exclusively, through the A1 receptor.

Stimulation via the A1 receptors causes transient activation of phospholipase C by a process that is inhibited by both cholera and pertussis toxins and markedly enhanced by treatment with dexamethasone such that cells now secrete in response to adenine analogs. As dexamethasone increases the expression of the A1 receptor as well as the α and β subunits of several trimeric G proteins, it has been suggested that both the α and β subunits of G proteins contribute to the activation of these two phospholipases. The sustained activation of phospholipase D and protein kinase C probably accounts for the ability of adenine analogs to synergize secretory responses to antigen and other secretagogues. Activation of phospholipase D results in the formation of phosphatidic acid and, via phosphatidate phosphohydrolase, 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 and m3 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 

**Chemical stimulants of RBL-2H3 cells**

Stimulants that bypass early receptor-mediated stimulatory events include the Ca2+-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 [Ca2+]i and activation of protein kinase C but they do not induce the release of secretory granules. In combination, however, they stimulate secretion. Thapsigargin, which elevates [Ca2+]i, by blocking the uptake of Ca2+ into IP3-sensitive stores, also stimulates secretion but only at concentrations (>100 nmol/L) for in excess of those (10 nmol/L) required for the elevation of [Ca2+]i. While these studies suggest that an increase in [Ca2+]i, and in protein kinase C provide signals for secretion, it should be noted that high concentrations of Ca2+-ionophore stimulate phospholipase C and phospholipase D.

**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. The activation of the β isoforms through G-protein-coupled receptors and the tyrosine phosphorylation of the γ isoform of phospholipase C through activation of Lyn/Syk tyrosine kinases via FcεRI 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.

Mobilization of intracellular and extracellular calcium ions

Early studies established that degranulation of rat peritoneal mast cells is dependent on external Ca2+ and is associated with influx of Ca2+ (Ca2+) and other divalent cations. 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 in which there is rapid release of Ca2+ from inositol 1,4,5-trisphosphate-sensitive Ca2+-stores followed by an influx of Ca2+. This influx is closely associated with the emptying of inositol 1,4,5-trisphosphate-sensitive Ca2+-...
stores\textsuperscript{51,52} and the generation of a diffusible messenger molecule\textsuperscript{53,54} from intracellular organelles. Influx is thought to occur through a low conductance current, designated \( I_{\text{crac}} \), for 'calcium release-activated calcium current', which has been characterized in mast cells\textsuperscript{55} and RBL-2H3 cells.\textsuperscript{56} This current appears to be highly selective for Ca\textsuperscript{2+} ions.\textsuperscript{55}

In the RBL-2H3 cell, antigen stimulation causes, after a short delay, a transient increase in [Ca\textsuperscript{2+}], in the absence of external Ca\textsuperscript{2+}\textsuperscript{51,57,58} which has been attributed to the release of Ca\textsuperscript{2+} from intracellular stores by inositol 1,4,5-trisphosphate.\textsuperscript{31,33} Stimulation in the presence of external calcium results in a sustained increase in [Ca\textsuperscript{2+}]. The sustained elevation in [Ca\textsuperscript{2+}], as determined by Ca\textsuperscript{2+}-sensitive fluorescent probes, is totally dependent on the influx of external Ca\textsuperscript{2+}.\textsuperscript{59} The increase in [Ca\textsuperscript{2+}], is associated with an increase in total intracellular Ca\textsuperscript{2+} possibly due to the uptake of cytosolic Ca\textsuperscript{2+} into mitochondrial stores when [Ca\textsuperscript{2+}] is elevated above basal levels.\textsuperscript{44,60} Other cations impede Ca\textsuperscript{2+}-uptake, either by blocking the entry of Ca\textsuperscript{2+} at the cell surface or by competing for Ca\textsuperscript{2+} entry into the cell.\textsuperscript{60} Entry of Ca\textsuperscript{2+} is suppressed also when cells are depolarized by high concentrations of external K\textsuperscript{+}.\textsuperscript{61,62} and a repolarizing current may be required to maintain influx.\textsuperscript{62-67} In addition to \( I_{\text{crac}} \), novel sphingolipid-gated Ca\textsuperscript{2+}-gated efflux channel (from Ca\textsuperscript{2+}-storage organelles) has recently been described in RBL-2H3 cells\textsuperscript{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\textsuperscript{2+}], 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 (\( \alpha \), \( \beta \), \( \gamma \), \( \delta \)), novel or calcium-independent (\( \epsilon \), \( \zeta \), \( \eta \), and \( \mu \)), and atypical (\( \xi \) and \( \chi \)) 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.\textsuperscript{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.\textsuperscript{70,71} Indeed, this is strongly supported by studies in RBL-2H3 cells.

RBL-2H3 cells contain the Ca\textsuperscript{2+}-dependent \( \alpha \), \( \beta \), and \( \gamma \) isoforms and the Ca\textsuperscript{2+}-independent \( \delta \), \( \epsilon \), and \( \zeta \) isoforms of protein kinase C.\textsuperscript{72-74} When cells are stimulated with antigen, these isoforms rapidly associate to variable extents (i.e. \( \delta \) the most and \( \zeta \) the least) with the membrane fraction but without external Ca\textsuperscript{2+}, only the Ca\textsuperscript{2+}-independent isoforms do so.\textsuperscript{72} Washed permeabilized cells lose all isoforms of protein kinase C, and do not secrete in response to antigen and carbachol,\textsuperscript{72} but stimulatory signals such as hydrolysis of inositol phospholipids are enhanced by as much as two- to three-fold.\textsuperscript{75}

Reconstitution of antigen-induced responses by provision of recombinant isoforms of protein kinase C to washed permeabilized cells have suggested that antigen-induced secretion of granules is mediated primarily by protein kinase \( \zeta \) and \( \delta \) and feedback inhibition of phospholipase C is mediated primarily by protein kinase \( \epsilon \).\textsuperscript{75} Similar studies have indicated that in antigen-stimulated cells, protein kinase \( \zeta \) and \( \epsilon \) transduce signals for the expression of the \( c-fos \) and \( c-jun \)\textsuperscript{76} and that protein kinase \( \delta \), by phosphorylating the \( \gamma \) subunit of Fc\textsubscript{RI} specifically, may promote endocytosis of the receptor.\textsuperscript{73} The isoforms also exhibit different rates of degradation when RBL-2H3 cells are continuously exposed to phorbol 12-myristate 13-acetate. Protein kinase C\( \beta \) and \( \alpha \) are degraded within minutes and hours respectively, whereas the Ca\textsuperscript{2+}-independent isoforms (\( \delta \), \( \epsilon \), and \( \zeta \)) resist degradation.\textsuperscript{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.\textsuperscript{79-81} Of the many drugs that we have tested, only the Ro series of protein kinase C inhibitors exhibit such selectivity.\textsuperscript{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\textsubscript{2} pathway.\textsuperscript{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 Sas, to convert Ras (p21\textsubscript{ras}) to its active GTP-bound state\textsuperscript{84-86} which, in turn activates the MAP kinase pathway via Raf\textsubscript{1}.\textsuperscript{87} Activation is accomplished through Ras-mediated translocation of the kinase Raf\textsubscript{1}, which when phosphorylated by an unidentified kinase, phosphorylates and activates a unique tyrosine/threonine kinase, MEK, which then phosphorylates and activates MAP kinase.\textsuperscript{88}

The cascade of events has been demonstrated in antigen-stimulated RBL-2H3 cells as indicated by the interactions of Shc and Sas with Grb2, the activation of Ras, and the phosphorylation of Shc.\textsuperscript{88} Raf\textsubscript{1}, MEK and p42\textsuperscript{mapk} are involved in a vaccinia expression system, has indicated that Syk is essential for activation of the Shc/Grb2/Sos\textsuperscript{89} and MAP kinase\textsuperscript{72} pathways when cells are stimulated by antigen.

The MAP kinase pathway may be activated in RBL-2H3 cells
through alternate pathways. Activation of the Raf/Mek/MAP kinase cascade via the muscarinic m1 receptors, for example, is not dependent on Syk. Current studies suggest that elevated [Ca\(^{2+}\)], 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.

**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\(^{59}\) 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 secretagogues.\(^{81,93}\) 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, 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 and patch-clamped mast cells.\(^{97}\) Because elevation in [Ca\(^{2+}\)], eliminated the requirement for the GTP analog, and vice versa, it was hypothesized that a Ca\(^{2+}\)-receptor (called G\(_{e}\), 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\(_{13}\), 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 active product of the H-ras oncogene\(^{100}\) or peptide analogs of Rab3a\(^{101}\) suggest 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\(^{2+}\)], 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\(^{2+}\)], 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. 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 C\(_{B}\) or the Ca\(^{2+}\)-independent protein kinase C\(_{S}\) (other isozymes were much less effective), but only in the presence of 1 \(\mu\)mol/L free Ca\(^{2+}\) to indicate separate roles for Ca\(^{2+}\) and protein kinase C in exocytosis.\(^{73}\)

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-acetylsn-glycero (OAG), and protein kinase C\(_{S}\), which does not require calcium for activation in the presence of various [Ca\(^{2+}\)]. Control experiments indicated that the elevation of [Ca\(^{2+}\)], only did not stimulate secretion even when [Ca\(^{2+}\)] was raised to 10 \(\mu\)mol/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 C\(_{S}\), physiologic concentrations of calcium (0.1 \(\mu\)mol/L and 1 \(\mu\)mol/L) induced no or little (>3%) secretion, but high [Ca\(^{2+}\)] induced moderate secretion possibly as a consequence of the stimulation of lipid metabolism (Fig. 2a). In the presence of 10 \(\mu\)mol/L OAG and protein kinase C\(_{S}\), a small elevation of [Ca\(^{2+}\)] 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\(^{2+}\)], and near maximal response at 700–1000 nmol/L [Ca\(^{2+}\)] (Fig. 2b), responses that were comparable to those observed in intact antigen-stimulated cells.\(^{98,103}\)

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

Protein kinase C\(_{S}\) 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 C\(_{B}\) also transduces a signal for secretion in permeabilized cells and its translocation to the membrane is dependent on calcium.\(^{72}\) As protein kinase C\(_{B}\) is the most potent of the two isoforms in promoting secretion,\(^{77}\) 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, [Ca²⁺]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 [Ca²⁺]ᵢ was the same for either isoform (Fig. 3a). Fifty percent of the maximal secretory response to antigen (EC₅₀) was observed with 190–210 nmol/L [Ca²⁺]ᵢ, and near maximal response, with 400–600 nmol/L [Ca²⁺]ᵢ (Fig. 3a). These values corresponded closely to those obtained in the studies with OAG (Fig. 2).

The second experiment was designed to assess the requirement for [Ca²⁺]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 [Ca²⁺]ᵢ 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 [Ca²⁺]ᵢ for activation of protein kinase Cβ was less than that for secretion (Fig. 3a,b). Half-maximal (EC₅₀) and near maximal responses were achieved with 110 nmol/L and 250 nmol/L [Ca²⁺]ᵢ, respectively. Thus, relatively small increases [Ca²⁺]ᵢ were required for activation of the β isofom.

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 [Ca²⁺]ᵢ and to different concentrations of protein kinase Cβ or δ before the addition of antigen. Secration was then initiated by raising [Ca²⁺]ᵢ to 1 μmol/L. Secreation was essentially complete within 10 min in the presence of 10 nmol/L protein kinase Cβ.
Fig. 3 (a) Calcium requirement for antigen-stimulated release of hexosaminidase by washed permeabilized cells incubated (15 min) with 100 nmol/L protein kinase Cγ (□), 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 Cγ, protein kinase Cβ 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, 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 [Ca²⁺], and activation of protein kinase Cβ or δ provide sufficient signals for secretion. Relatively modest increases in [Ca²⁺], 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 is now thought to be the high molecular weight PLA₂, usually referred to as cytosolic PLA₂ (cPLA₂), that has cDNA that has been cloned from human monoblast and murine macrophage cell lines. The deduced amino-acid sequence (85 kDa) indicates exceptional similarity between the human and murine forms of the enzyme, a shared Ca²⁺-dependent phospholipid binding domain and a single serine-containing consensus site for phosphorylation by MAP kinase. As noted earlier, activation of this pathway is achieved by sequential activation of Ras, Raf1, MEK1, and MAP kinases. Activation of this cascade of reactions is thought to result in the activation of cPLA₂ 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, cPLA₂ as well as the release of arachidonic acid. 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. These and other findings indicate that release of arachidonic acid is attributable exclusively to the regulation of cPLA₂ by MAP kinase (for activation of cPLA₂) and Ca²⁺ (for association of cPLA₂ with the membrane). 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 cPLA₂ 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
the activation of MAP kinase and cPLA₂ 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 cPLA₂ via Ras and MAP kinase.²³

**Synthesis and release of cytokines**

TNF-α is associated with discharged granules in human¹⁰⁹ and rodent¹¹⁰-¹¹² 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 FcεRI is probably dependent on the mobilization of Ca²⁺ 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 FcεRI-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 transcription¹⁷,¹¹⁵ or stabilization of short-lived mRNA transcripts¹¹⁶ by Ca²⁺-dependent kinases.¹¹⁷

Studies in RBL-2H3 cells²⁴ 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\textsuperscript{2+}], 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\textsuperscript{2+} and protein kinase C. Studies with various stimulants and inhibitors have indicated that simultaneous mobilization of Ca\textsuperscript{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 Raf\textsuperscript{118} or MAP kinase\textsuperscript{119} enhance the expression of a variety of cytokine genes in T cells and macrophages, the inactivation of IkB\textsuperscript{120} and enhanced binding activity of cytokine transcription factors, including NF-κB and AP1.\textsuperscript{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\textsubscript{2}, is regulated by MAP kinase and that both processes are dependent on modest increases in [Ca\textsuperscript{2+}]. The communicating links between FceRI 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\textsuperscript{2+}], for secretion and 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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