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Quercetin Sensitizes RBL-2H3 Cells to Polybasic Mast Cell Secretagogues Through Increased Expression of Gᵢ GTP-Binding Proteins Linked to a Phospholipase C Signaling Pathway

Jan Senyshyn, Rudolf A. Baumgartner, and Michael A. Beaven

Polybasic secretagogues such as mastoparan, compound 48/80, substance P, and somatostatin stimulate secretion in rat peritoneal mast cells through direct activation of the heterotrimeric G protein, G₁₃. Cultured RBL-2H3 mast cells do not normally respond to these secretagogues, but, as reported here, they do so after prolonged exposure to the kinase inhibitor, quercetin. This inhibitor, which causes phenotypic changes in RBL-2H3 cells, induces a substantial increase (more than sevenfold) in the expression of α subunits of the pertussis toxin-sensitive G proteins, Gₛ₂ and Gₛ₃. Compound 48/80-induced secretion is associated with transient hydrolysis of phosphoinositides and a transient increase in cytosolic calcium ions. These responses are inhibited by pertussis toxin, and in addition, secretion is blocked by calcium chelation and the protein kinase C inhibitor, Ro31-7549. These results delineate a pathway for compound 48/80-induced secretion in mast cells via G protein(s), phospholipase C, calcium, and protein kinase C. The results also imply that phospholipase C, most likely phospholipase Cβ3, can be transiently activated in RBL-2H3 cells by subunits of G protein subunits to induce cellular responses. The Journal of Immunology, 1998, 160: 5136–5144.

The early discoveries that compound 48/80 and other basic histamine secretagogues induced histamine release from tissues (1), disruption of mast cells (2), and anaphylactic-type reactions, which correlated with the extent of mast cell degranulation and histamine release (reviewed in Ref. 3), revealed that the mast cell was a likely mediator of immediate hypersensitivity reactions. These secretagogues were shown to cause the release of histamine and lipid inflammatory factors (1), then described as slow reacting substance of anaphylaxis (4, 5) but subsequently shown to be a mixture of leukotrienes (6). Compound 48/80, because of its potency, became an experimental exemplar of this category of secretagogues which also included peptides such as substance P, vasoactive intestinal peptide (VIP), somatostatin, and mastoparan. By various criteria, however, these secretagogues did not appear to act through specific membrane receptors but, rather, through some other mechanism (reviewed in Ref. 7).

The mechanism of action of these agents remained an enigma until relatively recently when it was reported that they directly stimulated GTPase activity of pertussis toxin-sensitive G, and G, trimeric G proteins (8–11). It was unclear whether the activated G proteins generated necessary signals for secretion or acted at late stages of the secretory process. Some of these compounds, for example, were shown to stimulate pertussis toxin-sensitive hydrolysis of phosphoinositides, release of intracellular calcium, and influx of external calcium in rat peritoneal mast cells (11–13).

Other evidence suggested that a pertussis toxin-sensitive G protein, most likely Gₛ₃, was directly activated by compound 48/80 in the plasma membrane of rat peritoneal mast cells and mediated a late step in exocytosis (10). For reasons that are obscure, only certain subtypes of mast cells, for example rat peritoneal mast cells, are activated by basic secretagogues, while other subtypes of mast cells, for example rat mucosal mast cells and cultured RBL-2H3 cells, do not respond to compound 48/80 (7, 14).

Ag stimulation of mast cells and cultured mast cell lines, such as RBL-2H3 cells, is normally associated with recruitment of the cytosolic tyrosine kinases, Lyn and Syk, by FcεRI (15–17). At least two signaling cascades are activated through Syk: the PLC/protein kinase C cascade for secretion of granules (18) and the ERK cascade for the activation of phospholipase A₂ and the release of arachidonic acid (19). These same cascades, however, can be activated via G proteins in RBL-2H3 cells made to express the G protein-coupled muscarinic m1 receptor (RBL-2H3 m1 cells) by stimulating these cells with the muscarinic agonist, carbachol (19). Secretion induced by either receptor is dependent on protein kinase C and an increase in [Ca²⁺], and is blocked by inhibitors of protein kinase C and calcium chelators (18, 20) or is potentiated by the protein kinase C agonist PMA (21). The generation of arachidonic acid and the cytokine TNF-α is dependent primarily on increased [Ca²⁺], and activation of mitogen-activated protein kinase (22, 23), although the release of both is influenced by protein kinase C (24, 25).

On the basis of biochemical criteria (26) and resistance to basic secretagogues, RBL-2H3 cells most resemble rat mucosal mast cells rather than rat peritoneal mast cells. RBL-2H3 cells become responsive to the polybasic secretagogues and show other phenotypic changes after coculture with NIH-3T3 fibroblasts (14). A change in phenotype can also be induced by prolonged exposure of RBL-2H3 cells to the kinase inhibitor, quercetin (27), which leads...
to increased amounts of intracellular histamine as well as increased numbers of electron-dense granules that stain with toluidine blue and safranin (28). Because these characteristics are similar to those of granules in rat peritoneal mast cells, RBL-2H3 cells appear to shift toward the phenotype of rat peritoneal mast cells.

As reported here, while investigating the changes induced by quercetin in RBL-2H3 cells and transfected RBL-2H3(m1) cells, we discovered that these cells become responsive to the polybasic secretagogues and show markedly increased expression of the G proteins, Gαq and Gqα. These changes allowed us to investigate the exact mode of action of these secretagogues in these cells. The secretagogues acted in a reversible manner, via pertussis toxin-sensitive G proteins, to cause transient, but substantial, hydrolysis of inositol phosphates and increases in [Ca2+]i, associated with rapid secretion of granules and release of arachidonic acid.

Materials and Methods

Reagents

These were obtained from the following sources: carbachol from Aldrich (Milwaukee, WI); quercetin, compound 48/80, and (+)sinuphrynzalone from Sigma (St. Louis, MO); somatostatin (catalogue no. PSOM10) from Bachem (Torrance, CA); VIP (catalogue no. 676385) from Calbiochem (La Jolla, CA); substance P (catalogue no. 152077) and mastoparan (catalogue no. 151587) from ICN (Costa Mesa, CA); pertussis and cholera toxins from List Biologics (Campbell, CA); Factor-test mTNF-α ELISA kit from Genzyme (Cambridge, MA); acetylmethylester and the free form of fura-2, pluronid acid, from Molecular Probes (Eugene, OR). Ro31-7549 was a gift from Dr. Koji Yamada, Tsukuba Research Laboratories (Eisai, Ibaraki, Japan). Radiolabeled compounds were obtained from DuPont-New England Nuclear (Boston, MA). Primary Abs included AS (for Gαi2 and Gγ1) and EC (for Gαqα), gifts from Dr. Paul Goldsmith (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health); Abs against PLCβ isofoms from Santa Cruz Biotechnologies (Santa Cruz, CA); and- rat Golgi complex (catalogue no. 1869) from American Type Culture Collection (Rockville, MD); and anti-rat mast cell pro tease II from Moredun Animal Health (Midlothian, Scotland). Reagents for immunocytofluorescence studies included indocarbocyanine (Cy3)-conjugated goat anti-rabbit IgG (H+L) (catalogue no. 111-165-144), FITC-conjugated donkey anti-sheep IgG (H+L) (catalogue no. 713-095-147), and FITC-conjugated goat anti-mouse IgG (H+L) (catalogue no. 115-095-146) from Jackson ImmunoResearch Laboratories (West Grove, PA), and, for fluorescence microscopy, fluorescein-conjugated wheat-germ agglutinin from Vector Laboratories (Burlingame, CA). The Ag DNP-BSA, and DNP-specific monoclonal IgE were supplied by Dr. Henry Metzger (National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health). Other reagents and materials were obtained from the sources listed previously. Stimulants and inhibitors were dissolved in DMSO, except for quercetin, which was dissolved in propylene glycol, and DNP-BSA and carbachol, which were dissolved in buffer. Dilutions were made directly into the required buffer so that the concentration of organic solvent was <0.1% (v/v). Control solutions contained the same concentration of vehicle.

Cell culture

RBL-2H3 and RBL-2H3(m1) cell lines were maintained in complete growth medium (MEM) supplemented with 13% FCS, glutamine, and antibiotic and antimycotic agents. Trypsinized cells were plated into culture dishes or multiwell cluster plates and were incubated overnight in complete growth medium with DNP-specific IgE (0.5 μg/ml) and radiolabeled reagents as required. Cultures were exposed to pertussis toxin (0.2 μg/ml) or cholera toxin (1 μg/ml) for 4 h according to previous protocols (31) where indicated. For each experiment, cells were washed the next day and medium was replaced with a glucose-saline, PIPES-buffered medium (25 mM PIPES (pH 7.2), 159 mM NaCl, 5 mM KCl, 0.4 mM MgCl2, 1.0 mM CaCl2, and 5.6 mM glucose). As noted, this medium was modified by buffering free calcium with EGTA (29). Where indicated, 10 μM of the protein kinase C inhibitor, Ro31-7549, was added 10 min before addition of stimulant. The concentrations of drugs and periods of stimulation were as noted in the text.

Coculture with NIH-3T3 fibroblasts and treatment with quercetin

RBL-2H3(m1) cells were cocultures with NIH-3T3 cells as previously described (14), except that the RPMI medium for coculture was buffered with 25 mM HEPES to maintain pH at 7.4. For stimulation with quercetin, RBL-2H3(m1) cells were incubated for 2 days with 30 μM quercetin until stated otherwise. The quercetin was prepared as a stock solution (60 mM) in propylene glycol and diluted to the desired concentration in culture medium.

Measurements of hexosaminidase, serotonin, TNF-α, inositol phosphates, and arachidonic acid

All measurements were made with confluent cultures (24- or 48-well cluster plates), and values were corrected for spontaneous release (2–4%) unless stated otherwise (29, 30). Secretion of the release of hexosaminidase, a granule constituent (33), or of radiolabeled serotonin, which is incorporated into secretory granules when incubated with RBL-2H3 cells (32). Hexosaminidase or radiolabeled serotonin was assayed in medium and cells as described previously (18, 29), and both markers showed identical rates of release (data not shown). In some experiments, cultures were incubated overnight with [3H]serotonin (1 μCi/ml), [14C]arachidonic acid (0.1 μCi/ml), and [3H]myo-inositol (4 μCi/ml) for simultaneous measurement of secretion, release of arachidonic acid, and generation of inositol phosphates as described previously (29, 30). Values were expressed as a percentage of the intracellular hexosaminidase or radiolabel that was released into the external medium.

For measurement of TNF-α cells were stimulated for 20 min. TNF-α was assayed in lysates of whole cultures as described previously (24) by use of a murine TNF-α-ELISA kit that used a monoclonal hamster antimurine Ab that reacted with mouse or rat TNF-α and β.

Electrophoretic separation and immunoblotting of pertussis toxin-sensitive Gα proteins and PLCβ isofoms

The preparation of cell lysates for SDS-PAGE separation of proteins and the subsequent Western blotting of proteins were performed as described previously (19, 22). Separation was performed on 8 to 16% linear gradient gels. Western blotting was performed on nitrocellulose (Hybond-C, Amersham), or PVDF (Immobilon-P, Millipore) membranes. Proteins of interest were detected by use of horseradish-conjugated secondary antibodies and the enhanced chemiluminescence detection system (Amersham).

Immunocytochemical detection of Gαi2, Gαqβ, and other proteins

Cells (200 cells/mm2) were incubated in the presence of vehicle or 30 μM quercetin for 48 h in complete growth medium on sterile 10-well immunofluorescent slides (Telfon-coated, Polysciences, Warrington, PA). Cells were fixed in 0.1% (v/v) glutaraldehyde in PBS, fixed in ice-cold 4% paraformaldehyde (in PBS) for 7 min, and washed. The cells were then permeabilized in methanol at −70°C and washed before exposure to (2 h) to 20% goat serum to suppress nonspecific binding. The slides were exposed (4°C) to rabbit polyclonal antibodies to Gαqα (AS Ab, 1/100 dilution) and Gαi2 (EC Ab, 1/50 dilution) for 4 h and then to the second Ab (cyamine-3-conjugated goat anti-rabbit IgG, 1/200 dilution) for 1 h. The cell preparations were viewed through a rhodamine filter set.

In some experiments, the cell preparations were simultaneously exposed to the antibodies against the Gαqα proteins and to either sheep anti-rat mast cell protease II (1/500 dilution) followed by FITC-labeled anti-sheep IgG (1/50 dilution) or mouse anti-rat Golgi complex (1/2000 dilution) followed by FITC-labeled anti-mouse IgG (1/50 dilution, ). Some preparations were also treated with fluorescein-conjugated wheat-germ agglutinin (1 μg/ml) to stain Golgi membranes. These preparations were viewed through rhodamine and FITC filter sets.

Measurement of [Ca2+]i, in single cells

Cells were grown on glass coverslips (25 mm in diameter) at a density of 4000 cells/mm2 in culture dishes. The cells were incubated overnight in complete growth medium with DNP-specific IgE and, for measurement of secretion, with [3H]serotonin (0.3 μCi/ml) as described above. They were then loaded with fura-2/acetylmethyl ester (2 μM for 45 min) in the presence of pluronid acid as recommended by the manufacturer. The coverslips was placed in a Dvorak-Stottler chamber (Nicholson Precision Instruments, Gaithersburg, MD), and the system was heated at 37°C. A Teflon spacer was placed in the chamber to reduce the free space to 50 μl, and effluent was collected for assay of [3H]serotonin. Reagents were dissolved in Ca2+-containing or Ca2+-free medium (see above) that contained 0.25 mM sinuphrynzalone to minimize loss of fura-2. Fura-2 fluorescence
was measured at 510 nM (excitation at 340 and 380 nM), and fluorescence was expressed as a ratio of the fluorescence at the two excitation wavelengths. The values for $K_d$ of fura-2 (230 nM), $R_{max}$ and $R_{min}$, were determined in thin films containing buffers of different concentrations of Ca$^{2+}$ exactly as previously described (34). Fluorescence was determined in a Deltascan fluorometer with photon detectors (Photon Technology International, Princeton, NJ).

Coverslips should be washed in chromic acid and extensively rinsed in distilled water. Cells treated with quercetin tend to detach from unwashed slips, and residual cells respond less well to compound 48/80 than those on washed coverslips or plastic tissue culture dishes.

Results

Sensitization of RBL-2H3 cells to compound 48/80 and other polyanionic secretagogues

Sensitization of RBL-2H3 cells to compound 48/80 by coculture with NIH-3T3 fibroblasts yielded cells that responded to compound 48/80 with 20 to 30% release of a granule marker, hexosaminidase. As reported by others (14), maximal responses were observed after 7 days of coculture. Alternatively, sensitization could be achieved by incubating RBL-2H3 cells with quercetin alone. Sensitization was apparent after 1 day (Fig. 1A) and reached a maximum by 2 days (data not shown). At 1 (Fig. 1A) or 2 (Fig. 1B) days, a maximal secretory response to compound 48/80 was achieved with 30 μM quercetin (data not shown). Maximal secretory responses for most experiments varied from 20 to 30%, but ranged from as high as 35% to as little as 10% in a few experiments. The reasons for this variation were unclear. Similar results were obtained with parental RBL-2H3 cells and in transfected RBL-2H3(m1) cells (data not shown). For all remaining experiments, RBL-2H3(m1) cells were used for comparison of responses to compound 48/80. Ag, and carbachol and were exposed to 30 μM quercetin or vehicle (untreated cells) for 2 days.

The responses to compound 48/80, Ag, and carbachol were apparent only after washing the cells free of quercetin (Fig. 2, final set of columns). Unwashed cells failed to respond to any stimulant including compound 48/80 (Fig. 2, middle set of columns) due, presumably, to inhibition of protein kinase C and other kinases at this concentration of quercetin (22). All subsequent experiments were performed with cells washed free of quercetin, and the term untreated cultures refers to cultures that were exposed to vehicle but not quercetin.

Quercetin-treated RBL-2H3 cells responded not only to compound 48/80 but also to somatostatin, VIP, and substance P with 50% maximal responses ($EC_{50}$) at 3 μg/ml, 30 μM, 15 μM, and 60 μM, respectively (Fig. 3). These potencies were comparable to those reported for these secretagogues in rat peritoneal mast cells (7, 35) and cocultures of fibroblasts and RBL-2H3 cells (14) or bone marrow-derived mast cells (36).

Evidence that compound 48/80 acted through a $G_i$ protein

The secretory responses of quercetin-treated cells to compound 48/80 (Fig. 4) and substance P (data not shown) were inhibited to the same extent (~75%) in pertussis toxin-treated cells, whereas the responses to Ag and carbachol in untreated or quercetin-treated cells either were not affected or were minimally inhibited (Fig. 4). As these results suggested that compound 48/80 and substance P acted via a $G_i$ protein, the effects of quercetin treatment on expression of $G_{i_p}$ proteins were examined. Such treatment resulted in at least a sevenfold increase in the expression of $G_{i_p}$ proteins as determined by immunoblotting (Fig. 5A). Consistent with previous data (31), $G_{i_{1A}}$ was not detectable in parental RBL-2H3 cells and remained undetectable in quercetin-treated cells (data not shown).

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Acquired sensitivity to compound 48/80 in quercetin-treated RBL-2H3(m1) cells. RBL-2H3(m1) cultures were exposed to the indicated concentrations of quercetin for 24 h (A) or 48 h (B) in normal growth medium and then washed free of quercetin. The cultures were stimulated with 20 ng/ml DNP-BSA (Ag) or 10 μg/ml compound 48/80 (48/80) or left unstimulated (Unstim.) for 15 min for measurement of the percent release of hexosaminidase as described in Materials and Methods. Values are uncorrected for spontaneous release and are the mean ± SE of three or more cultures. The experiment is representative of three similar experiments.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Removal of quercetin is required to unmask its ability to sensitize cells to compound 48/80. Cultures of RBL-2H3(m1) cells were incubated in vehicle (Control) or 30 μM quercetin for 48 h in normal growth medium. Medium was replaced with HEPES-buffered saline solution that contained 30 μM quercetin (unwashed) or vehicle (washed). The cultures were then stimulated with 20 ng/ml DNP-BSA (Ag), 100 μM carbachol (CBC), or 10 μg/ml compound 48/80 (48/80) for 15 min for measurement of the percent release of hexosaminidase as described in Materials and Methods. Values are corrected for spontaneous release (~3%) and are the mean ± SE of three or more cultures. The experiment is representative of three similar experiments.
Immunofluorescence cytochemical examination of quercetin-treated cells also revealed increased expression of $\alpha_i-2$ (Fig. 5, compare C with B) and $\alpha_i-3$ (Fig. 5, compare E with D), at least in some cells, but no remarkable change in intracellular distribution of these two proteins compared with that in untreated cultures. Approximately one-third of the cells showed significantly enhanced fluorescence, indicating the possibility that increased expression of $\alpha_i$ proteins (and the response to compound 48/80) occurred in a fraction of the cell population. $\alpha_i-2$ was associated with large granule-like structures (Fig. 5, C and B). $\alpha_i-3$, in contrast, was associated with perinuclear structures and filamentous-like structures (Fig. 5, D and E). Neither protein appeared to be highly localized in the plasma membrane, although background fluorescence might have impaired detection of small but significant amounts of these proteins. The use of Golgi cytochemical markers suggested colocalization of some $\alpha_i-3$ with Golgi membranes (data not shown). Rat mucosal mast cell protease II, a secretory granule constituent (37), showed a similar distribution as $\alpha_i-2$ and increased expression in quercetin-treated cells (data not shown).

Compound 48/80-induced secretion is associated with transient pertussis toxin-sensitive activation of PLC

Compound 48/80 induced a rapid release of hexosaminidase compared with the responses to carbachol or Ag (Fig. 6A). Release
occurred after a delay of about 15 s, but was essentially complete by 2 min. Compound 48/80 also stimulated transient (within 100 s) and limited release of arachidonic acid (1.5 ± 0.1 vs 2.1 ± 0.2 and 4.6 ± 0.5% release in cells stimulated for 15 min with 20 ng/ml DNP-BSA and 100 μM carbachol, respectively; data from five experiments). Compound 48/80 failed to stimulate production of the cytokine, TNF-α (<10 ng/ml vs 70 ± 5 ng/ml with 20 ng/ml DNP-BSA) in quercetin-treated cultures. These results suggested that compound 48/80 generated sufficient signals for secretion of granules but failed to stimulate a necessary signal or sufficient signals for optimal release of arachidonic acid and TNF-α.

Additional experiments revealed that compound 48/80-induced secretion was associated with a pertussis toxin-sensitive generation of inositol phosphates. In cells labeled with [3H]myo-inositol and [3H]serotonin, a granule marker (32), serotonin release coincided with the accumulation of inositol phosphates (Fig. 6B), and both responses were equally brief in two other experiments (data not shown). As with secretion (e.g., Fig. 4), production of inositol phosphates was impaired in pertussis toxin-treated cells, with 40 to 95% reduction in both responses compared with those in nontoxin-treated cells (range of values for six experiments performed on separate occasions). In all these experiments, compound 48/80 failed to stimulate production of inositol phosphates in untreated cultures (i.e., not exposed to quercetin).

The above results suggested that PLC was activated in a pertussis toxin-sensitive manner by compound 48/80, most likely via subunits of G_i proteins. Unlike the G_αi proteins, however, expression of PLCβ isoforms was not increased in quercetin-treated cells. As reported by others (38), only the β3 isofrom of PLC was detectable by immunoblotting in RBL-2H3 cells, and only this form was present in quercetin-treated cells (PLCβ1, -2, and -4 were not detectable by immunoblotting; data not shown). Therefore, the acquired sensitivity to compound 48/80 could not be attributed in any obvious way to increased expression of PLCβ.

Compound 48/80-induced secretion is associated with a transient increase in [Ca^{2+}]_i.

In cells loaded with fura-2 and radiolabeled serotonin, compound 48/80 induced transient increases in [Ca^{2+}]_i as indicated by changes in fura-2 fluorescence and, associated with this increase, secretion of radiolabeled serotonin (Fig. 7A). Subsequent stimulation with carbachol induced more persistent increases in [Ca^{2+}]_i and secretion than stimulation with compound 48/80 (Fig. 7A). Almost all cells exhibited calcium responses to compound 48/80 (>85% of 100 cells examined in three experiments) with variable delays (15–90 s, average delay of 30 s) in the onset of these responses (Fig. 7A). In contrast, each cell responded to carbachol abruptly (within 15 s attributable to void space in perfusion apparatus; Fig. 7A). Untreated cells showed no calcium or secretory responses to compound 48/80 (125 cells in five different experiments; data not shown) but they responded normally to carbachol (data not shown but similar to responses depicted in Fig. 7A).

Additional experiments revealed rapid desensitization of RBL-2H3 cells to compound 48/80, but not to carbachol, and that compound 48/80 and carbachol released calcium from the same intracellular pool. In the presence of external calcium, a second exposure to compound 48/80 failed to elicit an increase in [Ca^{2+}]_i, whereas multiple exposures to carbachol resulted in undiminished calcium responses (Fig. 7B, left trace). In the absence of external calcium, a second exposure to compound 48/80 again failed to induce a calcium response. Subsequent exposure to carbachol, however, elicited a brief calcium response to indicate incomplete emptying of intracellular pools. A second application of carbachol, however, elicited a brief calcium response to indicate incomplete emptying of intracellular pools.
results indicated that compound 48/80 partially depleted releasable calcium stores, possibly as a consequence of rapid desensitization to compound 48/80 compared with responses to carbachol. In A, perfused fura-2-loaded cells on coverslips were examined for changes in $[\text{Ca}^{2+}]_i$ by fluorescence imaging and secretion by measurement of $[\text{14C}]$serotonin in the perfusate. Cells were incubated previously to label secretory granules. The horizontal bars indicate periods of perfusion of compound 48/80 (20 $\mu$g/ml) and carbachol (100 $\mu$M). Similar results were obtained in five other experiments. B shows changes in fura-2 fluorescence in cells repeatedly exposed to compound 48/80 (solid bars) and carbachol (hatched bars) in the presence and the absence (open bar) of calcium. These responses are typical of those observed in at least 10 experiments. Additional related experiments are described in the text.

Dependency of compound 48/80-induced secretion on protein kinase C and calcium

In quercetin-treated cells, reduction of external calcium ($[\text{Ca}^{2+}]_o$) to 200 nM or lower impaired the secretory response to both compound 48/80 and carbachol (Fig. 8). In fact, secretory responses to both compounds exhibited similar dependencies for calcium, with complete loss of secretory responses at $<50$ $\mu$M $[\text{Ca}^{2+}]_o$ (Fig. 8). Untreated cells exhibited a similar calcium dependency with respect to carbachol, but, as in previous experiments, these cells did not respond to compound 48/80 (Fig. 8, inset).

As in previous studies (18), Ag-induced (Fig. 9A) and carbachol-induced (data not shown) secretions were absolutely dependent on protein kinase C and could be blocked by the protein kinase C inhibitor, Ro31-7549. This inhibitor also abrogated the response to compound 48/80 in quercetin-treated cells (Fig. 9A). The secretory responses to Ag and compound 48/80 in quercetin-treated cells showed a modest increase in the presence of the protein kinase C stimulant, PMA (compare A with B in Fig. 9). These enhanced responses, however, were still fully blocked by Ro31-7549 (Fig. 9B).

Discussion

The mechanism of action of compound 48/80 (a mixture of polymers derived from $N$-methyl-$p$-methoxy-phenylethylamine) and other polybasic mast cell secretagogues has attracted the attention
of numerous investigators over the past 40 yr (reviewed in Ref. 7). These compounds act only on certain subtypes of mast cells, such as rat peritoneal mast cells, to induce the rapid release of inflammatory mediators. Basophils, rat mucosal mast cells, and mast cells from most human tissues except skin as well as cultured RBL-2H3 and murine bone marrow-derived mast cells are not activated by these compounds (14, 36, 39). Compound 48/80-induced secretion is nonlytic (40), partially calcium dependent (41), and reminiscent of exocytosis (42). It acts at the cell surface, because immobilized compound 48/80 retains secretagogue activity (43), but it does not exhibit saturable binding or dose-response relationships that are characteristic of binding to discrete surface receptors (see discussion in Ref. 7). Biophysical studies suggest, instead, that compound 48/80 partially penetrates the plasma membrane to interact with membrane proteins (Refs. 44 and 45 and citations therein), most likely Go or Gi, trimeric G proteins because pertussis toxin blocks compound 48/80-induced secretion (12, 46, 47). Compound 48/80 can directly stimulate GTPase activity of these G proteins when they are reconstituted in phospholipid vesicles (48). On the basis of studies with Abs and inhibitory peptides, plasma membrane Gi-3 has been identified as a potential target for compound 48/80 in rat peritoneal mast cells (10). It is unclear, however, why compound 48/80 activates only certain subtypes of mast cells.

Another unresolved issue is whether the secretagogue-activated Gi proteins act at a late step of exocytosis or generate necessary intermediate signals for secretion. It has been proposed, for example, that Gi-3 may subserve the function of a hypothetical G protein, called Gi (10), which is thought to mediate a late step in exocytosis (49). Compound 48/80, however, stimulates a pertussis-toxin sensitive breakdown of inositol phosphates and uptake of calcium ions in rat peritoneal mast cells (12) and induces an increase in [Ca^{2+}]_i (through release from intracellular stores and influx of external calcium ions (13). Such stimulation is also associated with a modest increase in levels of 1,2-syn-diacylglycerol, largely as a consequence of hydrolysis of phosphatidylcholine by phospholipase D (50). Therefore, secretion could be mediated by signals generated through increases in [Ca^{2+}]_i and the activation of protein kinase C by diacylglycerol (51). As far as we are aware, there has been no unequivocal demonstration that compound 48/80-induced secretion is dependent on protein kinase C, and the connecting link between Gi proteins and pertussis toxin-sensitive hydrolysis of phosphoinositides is not established in mast cells. Pertussis toxin-sensitive phosphoinositide hydrolysis has been attributed to stimulation of PLCβ isoforms by βγ subunits of Gi proteins (52), and presumably, βγ subunits liberated through activation of Gi proteins by the polybasic secretagogues perform this function. The present studies reveal that cultured RBL-2H3 cells can be made to respond to polybasic mast cell secretagogues by prior exposure to the kinase inhibitor, quercetin. In addition to the previously described phenotypic changes in RBL-2H3 cells (28), quercetin induces substantial increases in the expression of Gi-2 and Gi-3, but not that of PLCβ. As in rat peritoneal mast cells, the secretory response to compound 48/80 in RBL-2H3 cells is associated with pertussis toxin-sensitive hydrolysis of phosphoinositides. Of the pertussis-toxin sensitive G proteins (i.e., Gi and Giγ), only Gi-2 and Gi-3 are expressed in RBL-2H3 cells (31), and of the PLCβ isoforms, only PLCβ3 is detectable in these cells (Ref. 38 and this report). Although the increased expression of these Gi proteins probably accounts for the acquired sensitivity to compound 48/80, a caveat is that Gi-2 and Gi-3 are located primarily in granule-like structures and Golgi, respectively (J. Senyshyn, unpublished observations). We could not detect similar localization of these proteins in the plasma membrane (Fig. 5), although background fluorescence may have masked changes if they had occurred. Another caveat is that whereas enhancement of expression of Gi proteins was apparent in some, but not all, cells by fluorescence microscopy, measurements of calcium responses indicated that most (>85%) of the quercetin-treated cells responded to compound 48/80.

In analogy to compound 48/80, parental RBL-2H3 cells respond to adenosine analogues, through an adenosine A3 receptor (53), in a pertussis toxin-sensitive manner to exhibit transient activation of PLC and an increase in [Ca^{2+}]_i (54, 55). Following treatment with dexamethasone, these responses and the expression of Gi-2 and Gi-3 are increased two- to threefold (56). Thus, both compound 48/80 and agonists of the adenosine A3 receptor induce transient pertussis-sensitive PLC-mediated signals in RBL-2H3 cells. These findings suggest that Gi-induced activation of PLCβ3, if mediated through G protein βγ subunits, is transient, in contrast to the sustained activation of PLCγ by Ag (54, 57, 58). Of probable relevance, PLCβ3 is phosphorylated and negatively regulated by protein kinase C in RBL-2H3 cells (38), and stimulation of these cells, at least through adenosine A3 receptors, results in sustained activation of phospholipase D and protein kinase C (55).

Previous results indicate that a modest increase in [Ca^{2+}]_i, and activation of protein kinase C are sufficient signals for secretion in RBL-2H3 cells (18). Virtually complete suppression of Ag-induced secretion can be achieved by chelation of external calcium and inhibition of protein kinase C with Ro31-7549 (18), which lacks the undesired effects of other protein kinase C inhibitors (59). Compound 48/80-induced secretion is similarly inhibited by these maneuvers and thus appears dependent on the same two signals. A paradox is that transient increases in [Ca^{2+}]_i are observed in the presence or the absence of external calcium, at least under the conditions of our experiments (Fig. 7B). Although secretion is closely correlated with this increase in the presence of external calcium (Fig. 7A), the lack of secretion at low external calcium (Fig. 8) could imply that the cellular secretory mechanism senses an influx of external calcium differently from release from internal sources.

Finally, we note that the mechanism of action of quercetin has not been determined. This compound, normally regarded as an anti-inflammatory agent, reversibly inhibits stimulatory signals and release of inflammatory mediators in RBL-2H3 cells, mast cells (22, 28, 60, 61), and other types of immunologic/inflammatory cells (reviewed in Ref. 27). In malignant cells, it arrests cells at a late stage of the G1 phase of the cell cycle (62, 63) and promotes cell differentiation (64). In RBL-2H3 cells specifically, quercetin inhibits cell proliferation and induces changes reminiscent of increased mast cell maturation (28). As noted earlier, these changes include a marked increase in the expression of Gi proteins and an acquired responsiveness to secretagogues that are known to activate these proteins. These responses, however, are unmasked only upon removal of quercetin. Quercetin is a known inhibitor of tyrosine, serine/threonine (27, 65), and phosphoinositide (65, 66) kinases as well as of components of the ERK mitogen-activated protein kinase cascade (22). Inhibition of any of these enzyme systems by quercetin might be expected to impede receptor-mediated responses in RBL-2H3 cells over the short term (17) and induce changes in quiescent transformed cells over the long term. The long term changes have allowed us to define for the first time a pathway via Gi, PLC (most likely PLCβ3), calcium, and protein kinase C for the secretagogue activity of compound 48/80. As with Ag stimulation (18), influx of calcium and activation of protein kinase C appear to be essential signals for compound 48/80-induced secretion in RBL-2H3 cells (18). However, the data do not...
References


17. Beaven, M. A., and R. A. Baumgartner. 1996. Mitogen and antigen mobilize calcium by similar mech-


