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Multiple signaling pathways control stimulus–secretion coupling in rat peritoneal mast cells

(pertussis toxin/calcium/phorbol ester/cyclic AMP/guanine nucleotide-binding regulatory protein)

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ABSTRACT **Fura-2 and membrane capacitance measurements were performed to investigate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and secretory responses of rat peritoneal mast cells following secretagogue stimulation. Compound 48/80 and internally applied guanosine 5′-[γ-thio]triphosphate (GTP[γ-S]) induced transient rises in $[Ca^{2+}]_i$ and caused membrane capacitance increases as secretion occurred. The 48/80-induced Ca^{2+} transients and secretory responses were blocked by guanosine 5′-[β-thio]diphosphate and neomycin, indicating that inositolphospholipid breakdown mediated by guanine nucleotide-binding regulatory protein (G protein) plays an important role in stimulus–secretion coupling. However, pertussis toxin did not block Ca^{2+} transients induced by 48/80 or GTP[γ-S], whereas secretory responses were either abolished (48/80) or developed only after a considerable delay (GTP[γ-S]). Similar effects were obtained by perfusing cells with cAMP: (*i*) Ca^{2+} transients following stimulation with 48/80 remained unaffected by cAMP, but secretory responses were abolished; (*ii*) GTP[γ-S] induced normal Ca^{2+} transients and degranulation in the presence of cAMP. Pretreatment of mast cells with phorbol 12-myristate 13-acetate (PMA) abolished 48/80- and GTP[γ-S]-induced Ca^{2+} transients (but not inositol trisphosphate-induced Ca^{2+} transients), whereas secretion still occurred. At the same time, the Ca^{2+} requirement for secretion was reduced by PMA. These results indicate that secretion in mast cells is under control of an as yet unidentified signaling pathway that involves a G protein. This pathway is distinct from inositolphospholipid turnover and may provide the triggering mechanism for secretion, whereas the inositolphospholipid pathway serves to increase $[Ca^{2+}]_i$ and renders the secretory process more sensitive to $[Ca^{2+}]_i$ by activating protein kinase C. Persistent activation of protein kinase C through phorbol ester imposes negative feedback control on the inositolphospholipid pathway, whereas cAMP may inhibit the unidentified signaling pathway.**

Mast cells participate in early allergic and inflammatory reactions by liberating histamine and other chemical mediators. The response is normally initiated by bridging of specific receptors of the IgE-type by the corresponding antigen and can also be elicited by other secretagogues, including compound 48/80, substance P, and somatostatin. The cellular events that take place following stimulation of mast cells (1) include (*i*) breakdown of inositolphospholipids, (*ii*) transient increase of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), (*iii*) generation of arachidonic acid, and (*iv*) increase in cyclic nucleotides. It is not clear which of these stimulation pathways constitutes the trigger for secretion and whether they can be activated independently or, in the case of acting synergistically, to what extent they contribute to the control of the exocytotic process.

Another potent mast cell secretagogue is internally administered guanosine 5′-[γ-thio]triphosphate (GTP[γ-S]) (2, 3), a nonhydrolyzable analogue of GTP that irreversibly activates guanine nucleotide-binding regulatory proteins (G proteins). This finding is not surprising, since the signaling pathways mentioned above are likely to be under control of G proteins. In mast cells, GTP[γ-S] actions include the release of Ca^{2+} from internal stores (4), presumably mediated by inositol trisphosphate. Yet Ca^{2+} is not necessary for GTP[γ-S]-induced secretion, nor is increased $[Ca^{2+}]_i$ by itself, under physiological conditions, a sufficient stimulus for secretion (5), although sustained high $[Ca^{2+}]_i$ can enforce exocytosis (6).

Activation of protein kinase C is known to reduce the Ca^{2+} requirement for a number of cellular processes (7) and has been suggested to serve this purpose in mast cell degranulation. Phorbol esters, which activate protein kinase C, can mimic agonist-induced cellular responses in many cell types, but they fail to induce sizable histamine release from mast cells (8), although synergistic effects with additionally applied ionophores have been reported (8, 9). Arachidonic acid, which is also liberated upon physiological stimulation, has been suggested to be an important intermediate or effector in mast cell secretion (10). Arachidonate mobilization in mast cells appears to result primarily from activation of the phospholipase A_2 pathway (11), which is likely to be under control of a G protein. Yet another G protein is involved in the activation of adenylate cyclase, which results in elevated levels of cAMP. The role of cAMP in secretion remains to be determined, since facilitatory and inhibitory effects have been reported (12).

Previous work has indicated two different G proteins to be involved in stimulus–secretion coupling in mast cells (13). In the present investigation it was attempted to differentially activate or block signal-transduction pathways by taking advantage of various pharmacological agents. The whole-cell configuration of the patch-clamp technique makes it possible to introduce substances into the cell while measuring changes in $[Ca^{2+}]_i$ and secretory responses by using the fluorescent Ca^{2+} indicator dye fura-2 and the membrane capacitance measurement technique, respectively.

MATERIALS AND METHODS

Rat peritoneal mast cells were obtained and kept as described (14). Experiments were performed at 23–26°C in a Mg^{2+}-rich saline solution: 140 mM NaCl/2.5 mM KCl/2 mM $CaCl_2$/5 mM $MgCl_2$/11 mM glucose/10 mM Hepes·NaOH, pH 7.2. The solution for filling pipettes (intracellular solution) was 135 mM potassium glutamate/20 mM NaCl/1 mM $MgCl_2$/10 mM Hepes·NaOH/0.2 mM Na_2ATP/0.1 mM fura-2 pentapotassium salt (Molecular Probes), pH 7.2. Except for experi-

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Abbreviations: $[Ca^{2+}]_i$, intracellular concentration of free Ca^{2+}; GDP[β-S], guanosine 5′-[β-thio]diphosphate; GTP[γ-S], guanosine 5′-[γ-thio]triphosphate; G protein, guanine nucleotide-binding regulatory protein; PMA, phorbol 12-myristate 13-acetate.

ments with GTP[γ-S], GTP (300 μM) was always added to the intracellular solution to sustain the cells' ability to degranulate (14). GTP[γ-S] (kindly provided by F. Eckstein, Max-Planck-Institut für Experimentelle Medizine, Göttingen, F.R.G.) was used at 100 μM to speed up secretory responses (3). In some experiments the following drugs were added to the pipette solution: guanosine 5'-[β-thio]diphosphate (GDP-[β-S], 200 μM; Boehringer Mannheim), cAMP (5–50 μM; Sigma), neomycin (0.1–1 mM; Sigma), and inositol 1,4,5-trisphosphate (0.5 μM; Amersham).

Compound 48/80 (5 μg/ml; Sigma) was applied directly to the cell under investigation by pressure ejection from a second pipette. In experiments with pertussis toxin (different batches were kindly provided by G. Schultz, Institut für Pharmakologie, Berlin, and P. Gierschik, Pharmakologisches Institut, Heidelberg), mast cells were preincubated at 37°C for 2–8 hr with toxin at 1 μg/ml. In the case of phorbol ester, preincubation with phorbol 12-myristate 13-acetate (PMA, 100 nM; Sigma) was for 30 min at 37°C.

Whole-cell capacitance measurements were performed with a two-phase lock-in amplifier (15). $[Ca^{2+}]_i$ was measured with fura-2 (16) loaded by diffusion from the recording pipette. Fluorescence of fura-2 was excited alternately by light at 360 and 390 nm and $[Ca^{2+}]_i$ was calculated from the fluorescence ratio. The calibration procedure and details of the measurement have been described (4).

RESULTS

Secretion and Ca^{2+} Transients Induced by Compound 48/80 Are Blocked by GDP[β-S] and Neomycin. Fig. 1*A* illustrates a typical response of a mast cell stimulated with the secretagogue 48/80. Following stimulation, a transient rise in $[Ca^{2+}]_i$ was observed. Changes in $[Ca^{2+}]_i$ showed considerable variability in shape and amplitude (see also ref. 17) including multiple Ca^{2+} transients; occasionally, fast Ca^{2+} transients were followed by a sustained plateau of elevated $[Ca^{2+}]_i$. Both the fast transient and the sustained increase in $[Ca^{2+}]_i$ likely resulted from the release of inositol trisphosphate, which liberates Ca^{2+} from internal stores (18) and causes Ca^{2+} influx (19). Thereafter, the cell membrane capacitance increased as the cell degranulated. Changes in membrane capacitance reflect cell surface area, which increases as the membranes of mast cell granules add to the plasma membrane during exocytotic fusion (20).

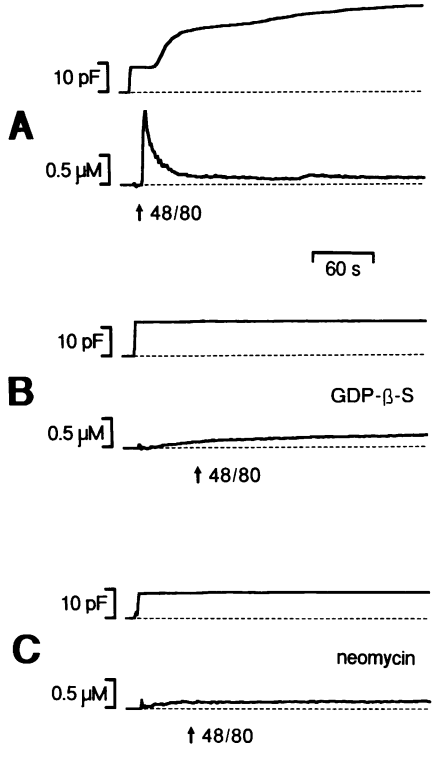

FIG. 1. Effects of GDP[β-S] and neomycin on secretory responses and $[Ca^{2+}]_i$. (*A*) Typical responses of a mast cell stimulated with 48/80 (5 μg/ml). (*B*) Cell perfused with internal solution containing GDP[β-S] (200 μM) and stimulated with 48/80. (*C*) Cell perfused with internal solution containing neomycin (1 mM) and stimulated with 48/80. Arrows indicate times of 48/80 application. Dashed lines indicate zero levels of capacitance and $[Ca^{2+}]_i$.

When recordings are made from mast cells in the whole-cell configuration, the cells rapidly lose their ability to degranulate in response to external stimulation by 48/80 (14, 21). This washout of secretion is due to the intracellular diminution of GTP, since the responsiveness for secretion can be maintained for some time by adding GTP to the internal solution (14). Interestingly, the generation of Ca^{2+} transients by 48/80 is not impeded by prolonged intracellular dialysis regardless of the absence or presence of GTP (14), raising some doubt that these Ca^{2+} transients are mediated by a G protein. This was tested by including GDP[β-S] in the pipette solution, which resulted in abolition of Ca^{2+} transients as well as secretion following stimulation by 48/80 (Fig. 1*B*; $n = 14$). For effective suppression of 48/80-induced Ca^{2+} transients, the stimulus had to be delivered >60–120 s after GDP[β-S] gained access to the cytosol, which probably reflects the time required for GDP[β-S] to diffuse from the pipette into the cell and displace GDP from its binding site on the G protein.

To ascertain that the 48/80-induced Ca^{2+} transients arise from inositolphospholipid breakdown, neomycin, which has been found to interfere with this process (13, 22), was perfused into the cell. Again, transient rises of $[Ca^{2+}]_i$ were suppressed (Fig. 1*C*; $n = 10$). As with GDP[β-S], a delayed application of 48/80 (40–90 s) was necessary to allow neomycin to take its action. The concentration of neomycin required to suppress Ca^{2+} transients was 1 mM, which also blocked 48/80-induced secretion. Neomycin at 100 μM usually did not prevent Ca^{2+} transients, yet secretory responses to 48/80 were attenuated. At this concentration of neomycin, the relative degranulation amplitude, calculated from the ratio of final to initial cell membrane capacitance, was found to be 1.8 ± 0.2 (mean ± SEM, $n = 14$) as compared to 2.3 ± 0.23 ($n = 14$) from controls obtained after identical 48/80 application delay of 60 s (ref. 14).

Pertussis Toxin Blocks 48/80-Induced Secretion But Not Ca^{2+} Transients. Pertussis toxin inhibits the functional coupling between activated receptors and certain G proteins by catalyzing ADP-ribosylation of the G protein's α subunit (23). Incubation of mast cells with pertussis toxin (1 μg/ml) for 2–8 hr at 37°C resulted in abolition of secretory responses following extracellular administration of 48/80 in all but 2 cells investigated. In contrast, Ca^{2+} transients were manifest in 32 out of 44 cells (Fig. 2*A*). This suggests that in the majority of cases the inositolphospholipid cascade remained operative while probably another G-protein-mediated second-messenger pathway important for stimulus–secretion coupling was blocked. Similarly, Ca^{2+} transients developed normally after intracellular application of GTP[γ-S], whereas secretory responses were delayed (Fig. 2*B*; $n = 9$). In some cells degranulation could not be traced to the end, since it had not terminated within 20–30 min.

cAMP Blocks 48/80-Induced Secretion But Not Ca^{2+} Transients. After antigenic stimulation of mast cells, a rapid increase in cyclic nucleotides is registered (24). Introduction of cAMP (50 μM) into the cell through the patch pipette did not induce secretion or Ca^{2+} transients. Regular Ca^{2+} transients could be induced by 48/80 or GTP[γ-S]. However,

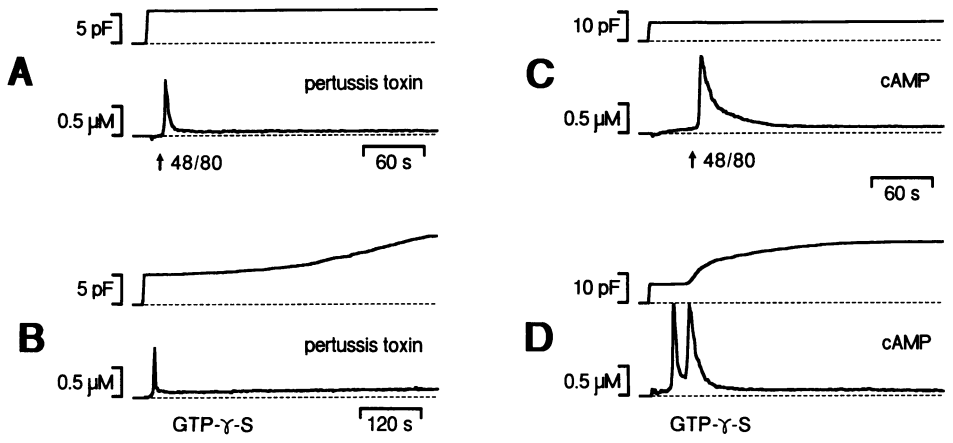

FIG. 2. Effects of pertussis toxin and cAMP on secretory responses and $[Ca^{2+}]_i$. (*A*) The cell was pretreated with pertussis toxin and was subsequently challenged with 48/80 at the time indicated. (*B*) Same as in *A*, except that the cell was stimulated internally with GTP[γ-S] (100 μM). (*C*) The cell was perfused with an internal solution containing cAMP (50 μM) and subsequently stimulated with 48/80 at the time indicated. (*D*) The cell was perfused with a solution that included GTP[γ-S] (100 μM) in addition to cAMP (50 μM).

whereas GTP[γ-S] was still able to elicit normal secretory responses (Fig. 2*D*; $n = 6$), they were completely suppressed following external stimulation by 48/80 (Fig. 2*C*; $n = 16$). When the intrapipette cAMP was lowered to 5 μM, secretory responses to 48/80 were no longer impeded ($n = 4$). Thus, the actions of cAMP on the cellular events monitored in this study were almost indistinguishable from those observed after cells were treated with pertussis toxin.

Phorbol Ester Blocks 48/80- and GTP[γ-S]-Induced Ca^{2+} Transients But Not Secretion. Protein kinase C is a Ca^{2+}/phospholipid-dependent enzyme that is physiologically activated by diacylglycerol resulting from the breakdown of inositolphospholipids (7). Phorbol esters can substitute for diacylglycerol by directly activating protein kinase C (26). When mast cells were incubated for 30 min at 37°C with 100 nM PMA, they underwent pronounced morphological changes. Within 60–90 min, most of the cells had acquired a flattened shape and pale appearance. Less than 10–20% of the cells showed signs of partial degranulation as judged by observation through the microscope.

When PMA-treated cells were stimulated with GTP[γ-S] they showed a normal secretory response (Fig. 3*A*) resulting in relative degranulation amplitudes of 3.8 ± 0.1 (mean ± SEM, $n = 6$). However, GTP[γ-S]-induced secretion was not accompanied by changes of $[Ca^{2+}]_i$. Similar results were obtained with 48/80 stimulation, which also was able to induce secretion in the absence of Ca^{2+} transients (Fig. 3*B*), although secretory responses to 48/80 were slower and smaller than in GTP[γ-S]-stimulated cells, yielding relative degranulation amplitudes of 1.6 ± 0.1 ($n = 8$) The mean relative degranulation amplitude of PMA-untreated control cells stimulated with 48/80 was 2.8 ± 0.2 ($n = 14$; ref. 14). The reduced amplitude of secretory responses to 48/80 may arise from effects of PMA treatment on receptor function (27).

The block of Ca^{2+} transients by PMA cannot be explained by a possible depletion of intracellular Ca^{2+} stores, since inositol trisphosphate was still able to induce increases in $[Ca^{2+}]_i$ (Fig. 3*C*; $n = 4$). This experiment also demonstrated that PMA-treated cells became more responsive to $[Ca^{2+}]_i$, so that some secretion occurred during inositol trisphosphate-induced increases in $[Ca^{2+}]_i$ of 0.5–1 μM. Without PMA treatment, a Ca^{2+}-only stimulus, in order to effect secretion, requires sustained, high $[Ca^{2+}]_i$ in the range of several micromolar (6).

DISCUSSION

The results presented here provide evidence for multiple regulatory mechanisms in mast cell secretion. The pharmacological actions on Ca^{2+} transients and degranulation of the substances investigated here are summarized in Table 1 and a model that illustrates the possible mechanisms of secretion control is depicted in Fig. 4. Secretagogue-induced Ca^{2+} transients are likely to result from the liberation of inositol trisphosphate and subsequent release of Ca^{2+} from intracellular stores, since similar Ca^{2+} transients are induced by inositol trisphosphate (18) and have been shown to occur in Ca^{2+}-free external solutions following antigenic or 48/80 stimulation (5). Some uncertainty that the Ca^{2+} transients are mediated by a G protein comes from the finding that mast cells perfused with GTP-free internal solutions for several minutes are still able to generate Ca^{2+} transients in response to 48/80, while secretory responses under these conditions are lost immediately due to GTP depletion (14). Compelling evidence that the actions of 48/80 are indeed mediated by a G protein (G_p) is provided by the fact that GDP[β-S] abolishes 48/80-induced Ca^{2+} transients as well as secretion. Thus, functioning G proteins are required for both the generation of Ca^{2+} transients and secretion, but different amounts of GTP

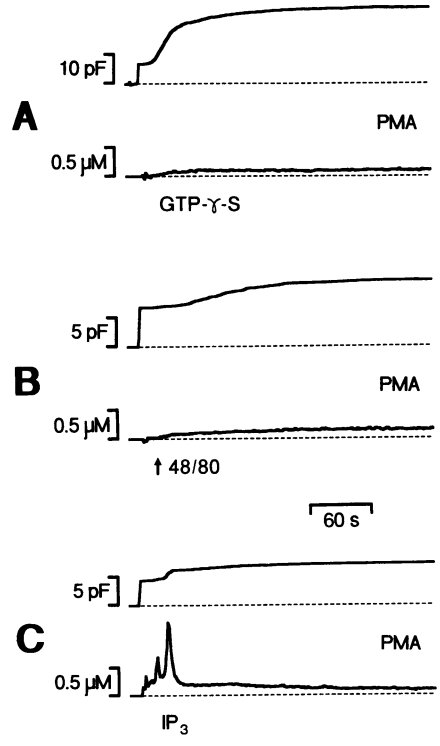

FIG. 3. Effects of phorbol ester on secretory responses and $[Ca^{2+}]_i$. Cells were pretreated with PMA. (*A*) The cell was challenged by internal perfusion with a pipette solution containing GTP[γ-S] (100 μM). (*B*) Compound 48/80 was used to externally stimulate the cell at the time indicated. (*C*) The cell was perfused with an internal solution supplemented with inositol 1,4,5-trisphosphate (IP_3, 1 μM).

Table 1. Effects of various agents on $[Ca^{2+}]_i$ and secretion

Agent	Ca^{2+} transient		Secretion	
	48/80	GTP[γ-S]	48/80	GTP[γ-S]
GDP[β-S]	−	ND	−	ND
Neomycin	−	ND	−	ND
Pertussis toxin	+*	+	−	+†
cAMP	+	+	−	+
PMA	−	−	+‡	+

−, Absence of Ca^{2+} transients or inhibition of secretion; +, normal responses; ND, no data.
*Present in 73% of the cells.
†Delayed and slow.
‡Reduced.

are needed for these two G-protein-mediated cellular functions. In the case of Ca^{2+} transients, the apparent independence of additionally provided GTP may be explained by phosphorylation of G-protein-bound GDP through a nucleoside diphosphokinase (28).

Neomycin (1 mM), which is believed to interact with membrane inositolphospholipids so that their breakdown by phospholipase C is impaired (13), prevented 48/80-induced Ca^{2+} transients and secretion. This suggests that phospholipid breakdown plays an important role in exocytosis. However, at a lower concentration of neomycin (100 μM), where regular-sized Ca^{2+} transients could be elicited by 48/80, secretion was reduced. This may indicate either that full-sized Ca^{2+} transients can occur with amounts of inositolphospholipid breakdown that are below the threshold required for maximal stimulation of secretion or that neomycin has additional inhibitory effects on processes that determine

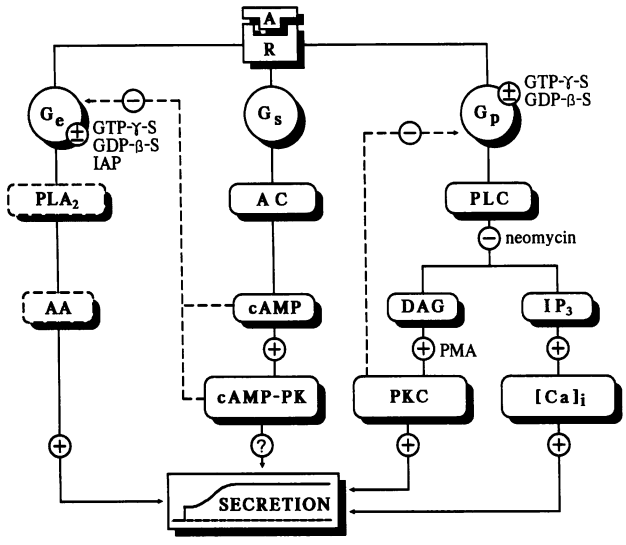

FIG. 4. Hypothetical model of the signaling pathways involved in stimulus–secretion coupling in mast cells and the presumed sites of action of various pharmacological manipulations. The model assumes three different G proteins (G_e, G_s, G_p) activated by receptor–agonist (R–A) interaction. The G proteins mediate the activation of membrane-associated enzymes [phospholipase A_2 (PLA_2), adenylate cyclase (AC), phospholipase C (PLC)], which in turn liberate second messengers [arachidonic acid (AA), cAMP, diacylglycerol (DAG), inositol trisphosphate (IP_3)] that act to activate another set of enzymes [cAMP-dependent protein kinase (cAMP-PK), protein kinase C (PKC)] or induce release of Ca^{2+} from internal stores. Inhibitory effects on the G_e pathway are provided by pertussis toxin (IAP) and cAMP (possibly via the cAMP-dependent protein kinase), whereas the G_p pathway is blocked by neomycin and recurrently inhibited by protein kinase C. The coupling between G_e and phospholipase A_2 is only tentative, as indicated by dashed boxes, and awaits investigations with pharmacological tools more selective than the ones currently available.

exocytosis. Since secretion in mast cells has been implied (13) to be under direct control of a G protein termed G_e (for exocytosis) and neomycin has been reported to interact with G proteins (29), the block of secretion induced by neomycin may not be solely related to inhibition of inositolphospholipid breakdown but possibly involves inhibition of G_e, too.

Pertussis toxin has been reported to inhibit phospholipid hydrolysis, arachidonate release, and histamine secretion from mast cells stimulated with 48/80 (23). The present study confirms the inhibitory effects of pertussis toxin on secretory responses induced by 48/80. However, Ca^{2+} transients following stimulation with 48/80 were still induced without delay in the majority of cells investigated, suggesting that the amount of inositol trisphosphate being released, albeit probably reduced, was still above the threshold for Ca^{2+} release. On the other hand, if the inositol trisphosphate/diacylglycerol pathway were solely governing control of exocytosis, one would have to assume that the concomitant generation of diacylglycerol after pertussis toxin is not sufficient for initiation of secretion. Alternatively, the differential effects of pertussis toxin on Ca^{2+} transients and secretion may be explained by the presence of an additional signal-transduction pathway that contains a G protein sensitive to pertussis toxin. In analogy to neutrophils (30), several lines of evidence suggest the presence of a G protein (G_e) that controls exocytosis in mast cells (13), although it is not clear at what stage G_e may act. A recently proposed model (13) suggested G_e to control secretion directly, at a site distal to G_p (the G protein mediating inositolphospholipid breakdown). Alternatively, G_e may be involved in a separate, parallel signaling pathway, possibly mediating activation of phospholipase A_2 with subsequent liberation of arachidonic acid. This pathway has been suggested to play an important role in secretion in a number of cells (see ref. 31) and has been shown to be affected by pertussis toxin in several preparations (32), including mast cells (23).

A recent patch-clamp study (33) suggested that pertussis toxin inhibits 48/80-induced secretion by acting on G_p, based on the finding that the toxin did not affect the time course of degranulation in GTP[γ-S]-stimulated cells. It was argued that GTP[γ-S] can still act through the pertussis toxin-insensitive G_e, whereas effects of 48/80 (supposedly mediated through G_p) are blocked by pertussis toxin. In the present study, however, stimulation of secretion by GTP[γ-S] was found to be markedly delayed by pretreatment with pertussis toxin, whereas Ca^{2+} transients were still manifest without delay. This is compatible with the finding that pertussis toxin does not in principle block the actions of GTP[γ-S] but rather delays them by about a factor of 5 (34). One possible reason for the discrepant results may be that different batches and different concentrations of pertussis toxin were employed. In the present investigation, pertussis toxin at 1 μg/ml inhibited GTP[γ-S] actions, whereas this may not have been the case with a toxin concentration of 180 ng/ml, although the toxin prevented secretion following 48/80 stimulation (33). This would suggest that the inhibitory effects of pertussis toxin on G_e may be more stringent with 48/80 stimulation and that higher concentrations or longer incubation periods are necessary to antagonize GTP[γ-S]-induced secretion.

The role of cAMP in mast cell secretion is a matter of considerable controversy (35). Most studies, however, assign an inhibitory effect on secretion, based mainly on the inhibitory actions of substances thought to elevate intracellular levels of cAMP (24). In this study, where cAMP was introduced directly into the cell, it was confirmed that high concentrations of cAMP are inhibitory to 48/80-induced secretion. The site of action of cAMP is not known. Inhibition of Ca^{2+} influx (36) or of Ca^{2+} release from internal stores (37) has been implicated as a possible mechanism of action. An

inhibition of Ca^{2+} influx by cAMP has recently been questioned by the finding that this second messenger may activate a chloride conductance and thereby hyperpolarize the membrane potential to support inositol trisphosphate-mediated Ca^{2+} influx in mast cells (19). The present study makes an inhibitory effect of cAMP on inositolphospholipid breakdown seem unlikely, since 48/80-induced Ca^{2+} transients were not impeded, whereas secretory responses were abolished.

The exact site of action of cAMP cannot be derived from the results presented here but, in principle, it could be located on the receptor or on the G protein. Unless one postulated differential phosphorylation of 48/80-receptor subtypes that couple independently to G_p and G_e, the receptor site appears unlikely since inositolphospholipid responses (through G_p) are transduced, as witnessed by Ca^{2+} transients. Therefore and because of the similarity to pertussis toxin, the site of action of cAMP may be tentatively placed on G_e. It is clear, however, that the mechanisms of action of pertussis toxin and cAMP are probably different (pertussis toxin ADP-ribosylates the α subunit of the G protein, whereas cAMP may serve to phosphorylate the G protein at a different site). This may explain the differences observed between cAMP and pertussis toxin with GTP[γ-S] stimulation. It should be noted, however, that the actions of pertussis toxin are not secondary to an elevation of cAMP levels, since these were found to be unaltered in toxin-treated mast cells (23).

The results obtained by pretreatment of mast cells with PMA add to the evidence that a G protein distinct from G_p may be involved in stimulus–secretion coupling in mast cells. In the majority of cells, activation of protein kinase C did not induce mast cell degranulation at basal $[Ca^{2+}]_i$. Restricted secretion in a few cells, possibly cells with slightly elevated $[Ca^{2+}]_i$, may add up to values comparable to those reported for phorbol ester-induced histamine release of about 10% (8, 38). In agreement with studies on rat basophilic leukemia cells (39, 40), agonist-induced responses of $[Ca^{2+}]_i$ were abolished after PMA pretreatment. Moreover, Ca^{2+} transients following stimulation with GTP[γ-S] were completely suppressed, yet it was possible to induce secretion by this stimulus. Stimulation with 48/80 also remained considerably effective in inducing secretion in the absence of Ca^{2+} transients, although secretory responses were smaller than those induced by GTP[γ-S].

Activation of protein kinase C through PMA has been shown to recurrently inhibit inositolphospholipid breakdown (25, 41). This negative feedback may occur at the receptor level, on the G protein, or on phospholipase C. An effect subsequent to the liberation of inositol trisphosphate can be ruled out, since inositol trisphosphate was still able to mobilize Ca^{2+} from internal stores. The fact that 48/80 can still induce secretion (even with high concentrations of PMA) suggests that the major effects of PMA occur distal to the receptor, possibly involving inhibition of G_p or phospholipase C. This does not rule out PMA effects on receptors. In fact, in the presence of high concentrations of PMA this down-regulation of receptors (27) may account for the somewhat smaller secretory response.

From the findings presented here it seems that multiple signaling pathways control stimulus–secretion coupling in mast cells. Secretion appears to be controlled by an as yet unidentified signaling pathway, which may involve the putative G protein G_e. The inositol trisphosphate/diacylglycerol pathway may support secretion by regulating $[Ca^{2+}]_i$ and reducing the apparent Ca^{2+} requirement for secretion. The model depicted in Fig. 4 assumes three parallel signaling pathways involving three different G proteins (G_e, G_s, G_p) and may be favored over the model that assumes G_p and G_e to act in series (13). According to the serial model, receptor stimulation should be ineffective in eliciting secretion through G_e under conditions where inositolphospholipid breakdown is blocked. However, contrary to such a prediction, cells pretreated with PMA, which suppresses inositolphospholipid breakdown (25, 41), still degranulated in response to 48/80 (see Fig. 3*B*). This is in agreement with studies in which 48/80- or antigen-induced secretion was not impeded in mast cells (8) and rat basophilic leukemia cells (39, 40) pretreated with low concentrations of phorbol ester, suggesting a parallel signaling pathway that can induce secretion independent of inositolphospholipid breakdown.

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