Calcium- and guanine-nucleotide-dependent exocytosis in permeabilized rat mast cells

Modulation by protein kinase C

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We have used a digitonin-permeabilized cell system to study the signal transduction pathways responsible for stimulus–secretion coupling in the rat peritoneal mast cell. Conditions were established for permeabilizing the mast cell plasma membrane without disrupting secretory vesicles. Exocytotic release of histamine from digitonin-permeabilized cells required a combination of micromolar concentrations of Ca^{2+} and the stable guanine nucleotide analogue guanosine 5'-[γ -thio]triphosphate (GTP[S]), but was independent of exogenous ATP. In the presence of 40 μM -GTP[S], exocytosis was half-maximal at 1.3 μM - Ca^{2+} and maximal at 10 μM - Ca^{2+} ; GTP[S] alone (100 μM) had no effect on histamine release in the absence of added Ca^{2+} . In the presence of 10 μM free Ca^{2+} , 5 μM -GTP[S] was required for half-maximal exocytosis. To examine the possible role of protein kinase C (PKC) in exocytosis, we utilized 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to activate PKC and studied its effect on histamine release from permeabilized mast cells. Cells that had been incubated with TPA (25 nM for 5 min) exhibited increased sensitivity to both GTP[S] and Ca^{2+} . The PKC inhibitor staurosporine blocked the effect of TPA without inhibiting normal exocytosis in response to the combination of GTP[S] and Ca^{2+} . In addition, down-regulation of mast-cell PKC by long-term TPA treatment (25 nM for 20 h) blocked the ability of the cells to respond to TPA and inhibited exocytosis in response to Ca^{2+} and GTP[S] by 40–50%. These results suggest that the sensitivity of the exocytotic machinery of the mast cell can be altered by PKC-catalysed phosphorylation events, but that activation of PKC is not required for exocytosis to occur.

INTRODUCTION

The signal transduction pathways responsible for stimulus–secretion coupling in mammalian cells are complex. The development of permeabilized cell systems [1–17] has permitted the biochemical analysis of these pathways. Early experiments suggested that an increase in the intracellular concentration of Ca^{2+} was sufficient to stimulate exocytosis, provided that cellular ATP levels were maintained [1,2,11,12]. More recently, several groups have reported a requirement for guanine nucleotides at some step in exocytosis [3–7, 13]. These observations have led to the proposal that an as-yet unidentified GTP-binding protein, termed G_E , may be an integral part of the signal transduction apparatus [18]. At the same time, it is well established that an early signal transduction event in many cell types is the activation of a phosphatidylinositol-specific phospholipase C (PI-PLC) by a receptor-linked GTP-binding protein, G_p (see [19] for a review). Activated PI-PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The role of IP_3 is to liberate intracellular Ca^{2+} stored in the endoplasmic reticulum

[20], whereas DAG serves to activate Ca^{2+} - and phospholipid-dependent protein kinase C [21].

In mast cells and blood basophils, stimulus–secretion coupling transduces the cross-linking of cell-surface IgE receptors to the exocytotic release of histamine [22]. The observation that IgE receptor cross-linking stimulated PIP_2 hydrolysis in both RBL-2H3 basophilic leukaemia cells and mast cells [23,24] suggested that the guanine nucleotide requirement for exocytosis in permeabilized mast cell systems might reflect a requirement for G_p activation. However, recent experiments by Cockcroft *et al.* [6] indicate that this simple explanation may not be sufficient. Using the aminoglycoside antibiotic neomycin to inhibit PI-PLC activity, these authors showed that PIP_2 hydrolysis is not a prerequisite for Ca^{2+} and GTP[S]-stimulated exocytosis in permeabilized mast cells. This finding suggests that the requirement for guanine nucleotides in exocytosis does not necessarily reflect a requirement for PI-PLC activation and strengthens the contention [18] that a second GTP-binding protein (G_E) is required for exocytosis in mast cells.

As noted above, protein kinase C is also involved in the Ca^{2+} -mobilizing signal-transduction pathway; however, its role in the regulation of exocytosis is still

Abbreviations used: LDH, lactate dehydrogenase; GTP[S], guanosine 5'-[γ -thio]triphosphate; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; 4 α -PDD, 4 α -phorbol 12,13-didecanoate; PI-PLC, phosphatidylinositol-specific phospholipase C; IP_3 , inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PIP_2 , phosphatidylinositol 4,5-bisphosphate; DMSO, dimethyl sulphoxide; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; SLO, streptolysin-O.

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unclear. PKC may exert both positive and negative modulatory effects, depending on the cell type and system. Activation of PKC has been shown to block the generation and maintenance of the Ca^{2+} signal [25], yet pretreatment of mast cells with TPA (12-*O*-tetradecanoylphorbol 13-acetate) (which activates PKC directly [26]) lowers the effective concentration of the Ca^{2+} ionophore A23187 required to elicit an exocytotic response [27,28]. Based on the latter results, it has been suggested that activation of PKC may positively modulate mast cell exocytosis *in vivo*; however, the precise step(s) in the stimulus–secretion pathway modulated by PKC remain unknown. For instance, PKC may modulate the sensitivity of exocytosis by altering the requirement for Ca^{2+} or guanine nucleotides. In the experiments reported here we address this question by examining the effect of TPA treatment on the threshold concentrations of Ca^{2+} and GTP[S] required for exocytosis. Our results suggest that while PKC activation is not required for exocytosis, PKC-catalysed phosphorylation does modulate the ability of both Ca^{2+} and guanine nucleotides to stimulate exocytosis.

EXPERIMENTAL PROCEDURES

Materials

Male Sprague–Dawley retired breeder rats were obtained from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.). Percoll was purchased from Pharmacia (Piscataway, NJ, U.S.A.). GTP[S] was from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). GTP[S] stocks were prepared at 1.0 mM in aliquots of mast-cell buffer (NaCl, 137 mM; KCl, 2.7 mM; glucose, 5.6 mM; bovine serum albumin, 1.0 mg/ml; Pipes 20 mM, pH 6.80) and stored at -80°C ; each aliquot was only used once. EGTA was purchased from Fluka Chemicals (Hauppauge, NY, U.S.A.) and from Sigma (St. Louis, MO, U.S.A.). Digitonin (Fisher D-58) was prepared as a 50 $\mu\text{g}/\text{ml}$ stock in mast-cell buffer and stored in aliquots at -20°C . TPA, 4 α -phorbol 12,13-didecanoate (4 α -PDD) and 4 α -phorbol (all from Sigma) stocks were prepared in 100% dimethylsulphoxide (DMSO) and stored at -20°C . Staurosporine was from Kamiya Biomedical Company (Thousand Oaks, CA, U.S.A.), and was prepared as a 2.0 mM stock in DMSO; working solutions of staurosporine were prepared just before use. *o*-Phthalaldehyde, RPMI-1640, Dulbecco's modified Eagle's medium (DMEM) and prestained molecular mass markers for SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) were from Sigma. Chemicals for SDS/PAGE were purchased from Bio-Rad Laboratories (Rockville Centre, NY, U.S.A.). Nitrocellulose membranes were from Hoefer Scientific Instruments (San Francisco, CA, U.S.A.). Anti-(protein kinase C) antiserum, non-immune IgY and rabbit anti-IgY antiserum were kindly supplied by Dr. Curtis Ashendel, Purdue University, West Lafayette, IN, U.S.A. Partially purified rat brain PKC was a gift from Dr. Lee Witters, Dartmouth Medical School. ^{125}I -labelled goat anti-rabbit IgG (whole molecule) was purchased from New England Nuclear (Wilmington, DE, U.S.A.). X-OMAT AR X-ray film was from Kodak (Rochester, NY, U.S.A.).

Mast cell purification

Mast cells were purified from male Sprague–Dawley retired breeder rats by centrifugation of peritoneal cells

in Percoll as described by Enerbäck & Svensson [29], with modifications. Briefly, rats were killed with ether and injected intraperitoneally with 20 ml of ice-cold phosphate-buffered saline (PBS; NaCl, 137.0 mM; Na_2HPO_4 , 8.0 mM; KCl, 2.7 mM; KH_2PO_4 , 1.5 mM; pH 7.30). Following 90 s of abdominal massage, peritoneal fluid was removed using a perforated 15 ml conical plastic tube (Falcon) and a plastic pipette. The mixed peritoneal cell suspension (approx 90% monocytes) was centrifuged for 5 min (250 *g*) at 4°C . Cell pellets (two per rat) were resuspended in 4.0 ml of iso-osmotic Percoll [NaCl, 137.0 mM; Na_2HPO_4 , 8.0 mM; KCl, 2.7 mM; KH_2PO_4 , 1.5 mM; Percoll, 90% (v/v); pH 7.3] and centrifuged (125 *g*) for 15 min at 4°C . Following aspiration of supernatants, cell pellets (> 90% mast cells as assessed by microscopy) were washed twice in ice-cold mast-cell buffer and resuspended at 5×10^5 cells/ml in the same buffer. Cell counts were performed using a Bright-line haemocytometer.

Permeabilization and histamine release

In a typical experiment, 50 μl of mast cell suspension at 5×10^5 cells/ml was added to 12 mm $\times$ 75 mm polypropylene tubes (prewarmed for 5 min to 37°C) containing 950 μl of buffer with the indicated concentrations of digitonin, Ca^{2+} (see below) and GTP[S], and incubated for 5 min at 37°C . Reactions were terminated by adding 1.0 ml of ice-cold PBS to each tube, vortexing and transferring the tubes to ice. Cell suspensions were centrifuged (250 *g*) for 5 min at 4°C ; 900 μl of supernatant was then removed from each tube and transferred to a new tube. A 100 μl portion of 100% (w/v) trichloroacetic acid was added to each tube and the tubes were centrifuged for 15 min (250 *g*) at 4°C . Supernatant (750 μl) was removed from each tube, and the histamine content was determined by using *o*-phthalaldehyde as described by Shore *et al.* [30]. In experiments with TPA, 50 μl of purified mast-cell suspension at 5×10^5 cells/ml was preincubated with 50 μl of mast-cell buffer containing either TPA or vehicle (0.1% DMSO) for 5 min at 37°C before addition of 900 μl of mast-cell buffer containing digitonin, GTP[S], and/or Ca^{2+} buffers as indicated; buffers were always warmed to 37°C before addition. The concentration of DMSO used ($\leq 0.1\%$) had no effect on histamine release. In the experiments shown in Fig. 6 and Table 1, 50 μl of mast-cell suspension at 5×10^5 cells/ml was incubated either with 50 μl of mast-cell buffer containing the indicated concentrations of staurosporine in DMSO, or with 50 μl of mast-cell buffer containing DMSO alone for 5 min at 37°C before incubation with 100 μl of mast-cell buffer containing sufficient TPA to raise the TPA concentration to the indicated value. Following an additional 5 min incubation at 37°C , 800 μl of mast-cell buffer containing sufficient digitonin, Ca^{2+} and/or GTP[S] to achieve the desired concentrations was added, and incubation was carried out for 5 min at 37°C . In the experiment shown in Table 2, purified mast cells were washed twice in ice-cold DMEM, then resuspended in warm DMEM (37°C , 5×10^5 cells/ml) containing either 25 nM-TPA or vehicle (0.05% DMSO). The cell suspensions were incubated in 8% CO_2 in air at 37°C for 20 h in Teflon jars (Scientific Specialties, Randallstown, MD, U.S.A.), then washed twice in ice-cold mast-cell buffer and assayed for histamine release as described above. In all experiments, histamine release is expressed

as a percentage of total cellular histamine; background release (in the absence of digitonin) was less than 9% in all experiments and has been subtracted from the values shown. Lactate dehydrogenase (LDH) release from permeabilized cells is expressed as a percentage of total cellular LDH activity.

Ca²⁺ buffers

Ca²⁺ buffers were prepared in mast-cell buffer (pH 6.8) containing 2 mM-EGTA, 2 mM-MgCl₂ and sufficient CaCl₂ to give the indicated free Ca²⁺ concentrations. Free Ca²⁺ concentrations were calculated using a computer program [31] and verified with a Ca²⁺-sensitive electrode (World Precision Instruments, New Haven, CT, U.S.A.). Ca²⁺ standard solutions were purchased from WPI, or prepared as described by Tsien & Rink [32]. In calculating the free Ca²⁺ concentrations of Ca²⁺ buffers, pH values were decreased by 0.11 to compensate for the fact that pH electrodes measure H⁺ activity whereas the stability constants for Ca²⁺-EGTA complexes are based on H⁺ concentration [32,33].

SDS/PAGE

All samples for SDS/PAGE were heated for 3 min at 100 °C before electrophoresis using the method of Laemmli [34] on 12% polyacrylamide minislab gels. Purified mast cells were incubated as described for PKC down-regulation except that the cells were cultured in 35 mm cell culture plates (Corning) in RPMI-1640 medium; following incubation, the plates were gently scraped with a rubber policeman to detach the cells. Cells were washed, pelleted and solubilized in hot SDS/PAGE sample buffer [34] containing 25 mM-dithiothreitol and 10 mM-EDTA to a final concentration of 1 × 10⁷ cell equivalents/ml. Samples of partially purified rat brain PKC were diluted into 2 × concentrated SDS/PAGE sample buffer containing 50 mM-dithiothreitol and 20 mM-EDTA before heating and electrophoresis. Pre-stained molecular mass standards used were β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (49 kDa), lactic dehydrogenase (37 kDa) and triosephosphate isomerase (27 kDa).

Immunoblotting

All blotting and incubation procedures were carried out at room temperature. After gel electrophoresis, proteins were electrophoretically transferred on to 0.45 μm pore size nitrocellulose sheets (Hoefer) in a Hoefer Transphor apparatus for 3 h at 350 mA. Nitrocellulose sheets were then incubated in blocking buffer [PBS containing 0.5% Tween-20, 0.02% NaN₃, and 5% (w/v) Carnation non-fat dry milk] for 1 h. Sheets were then transferred to either anti-PKC antiserum or non-immune IgY, each diluted 1:500 in blocking buffer, and incubated for 15 h. The anti-PKC antiserum was produced by immunizing chickens with rat brain PKC [35]; as the antiserum is prepared from egg yolk, it is designated IgY. Sheets were washed five times for 10 min each in PBS containing 0.5% Tween-20, reblocked with blocking buffer for 1 h, and then incubated with rabbit anti-IgY antiserum diluted 1:500 in blocking buffer for 1.5 h. Following an additional five washes and another reblocking step, ¹²⁵I-goat anti-rabbit IgG (whole molecule) diluted to 1.0 μCi/blot in blocking buffer was added and incubated for 2 h. After a final five washes,

the sheets were air-dried and exposed to Kodak X-OMAT AR X-ray film for 24–48 h at –80 °C using an intensifying screen.

Presentation of data

All experiments were carried out in duplicate and repeated a minimum of three times with different cell preparations.

RESULTS

Mast cell permeabilization

To determine the concentration of digitonin required to selectively permeabilize rat peritoneal mast cells, a defined number of purified cells (2.5 × 10⁴) was treated with increasing concentrations of digitonin and analysed for release of histamine (Fig. 1a) or of the cytosolic marker enzyme LDH (Fig. 1b). The results of this experiment demonstrate that selective permeabilization is possible: low concentrations of digitonin (< 6.0 μg/ml) released a large proportion of LDH, but only negligible amounts of histamine. Half-maximal release of LDH occurred at a digitonin concentration of 1.5–2.0 μg/ml, both without and with the addition of Ca²⁺ and GTP[S]. A concentration of 10 μg of digitonin/ml was required for complete release of histamine in the absence of a stimulus; in contrast, histamine release in the presence of 10 μM-Ca²⁺ and 40 μM-GTP[S] was complete at a digitonin concentration of 2 μg/ml. Based on these results, we chose to use a digitonin concentration of 4.0 μg/ml for subsequent experiments. Trypan Blue staining of cell preparations permeabilized with 4.0 μg of digitonin/ml revealed that > 90% of the cells were permeable to this low molecular mass marker (results not shown). The loss of 70–80% of cellular LDH (142 kDa) from cells treated with 4.0 μg of digitonin/ml is indicative of the large size of the lesions generated in mast cell plasma membranes. However, the occurrence of exocytosis in the face of such a large efflux of cytosolic proteins does not necessarily mean that cytosolic proteins are not required for any step in the exocytotic pathway since the stimuli (Ca²⁺ and GTP[S]) are present from the start of the permeabilization period.

Ca²⁺- and guanine-nucleotide-dependence of exocytosis

Howell *et al.* [7] have reported that mast cells permeabilized with the bacterial toxin streptolysin-O (SLO) exhibit an obligatory synergy between micromolar concentrations of Ca²⁺ and GTP[S] for exocytosis. With optimal concentrations of GTP[S], they report a Ca²⁺ threshold for exocytosis of 1–2 μM. Our results with digitonin-permeabilized mast cells confirm these observations (Fig. 2a). In the presence of 40 μM-GTP[S], histamine release is half-maximal at approx. 1.3 μM free Ca²⁺ and complete at 10 μM free Ca²⁺. In the absence of GTP[S], mast cells do not release histamine even at 32 μM-Ca²⁺ (pCa = 4.5). We next investigated the effects of titration of GTP[S] on the system in the presence and absence of 10 μM-Ca²⁺. As shown in Fig. 2(b), histamine release in the presence of 10 μM free Ca²⁺ is half-maximal at approx. 5.0 μM-GTP[S]; in the absence of Ca²⁺, exocytosis does not occur even in the presence of high (100 μM) concentrations of GTP[S]. These results are also in agreement with results obtained with SLO-permeabilized mast cells [6,7]. It is noteworthy that exocytosis stimulated by the combination of Ca²⁺ and

Fig. 1. Release of (a) histamine and (b) LDH from digitonin-permeabilized rat mast cells.

Purified mast cells (2.5×10^4 /tube) were incubated for 5 min at 37 °C in mast-cell buffer containing the indicated concentrations of digitonin as well as $10 \mu\text{M-Ca}^{2+}$ and $40 \mu\text{M-GTP[S]}$ (■, ▲) or 2.0 mM-EGTA (□, △). At the end of the incubation, release of histamine was determined using *o*-phthalaldehyde (a). In a separate experiment, release of LDH was determined (b). Both LDH and histamine release are expressed as a percentage of total cellular content. Unless otherwise stated, the experiments shown in this and subsequent Figures are representative samples drawn from sets of at least three replicate experiments with different cell preparations. Data points represent means $\pm$ range of duplicate observations, except where the range was smaller than the symbol used.

GTP[S] in the digitonin-permeabilized cells is not dependent on exogenous ATP.

Effects of TPA on exocytosis in permeabilized mast cells

To examine the possibility that PKC might modulate exocytosis in the permeabilized mast cell system, we tested the effects of preincubation with increasing

Fig. 2. Histamine release from digitonin-permeabilized rat mast cells as a function of Ca^{2+} and GTP[S] concentrations

(a) Purified mast cells (2.5×10^4 /tube) were incubated for 5 min at 37 °C in mast-cell buffer containing $4.0 \mu\text{g}$ of digitonin/ml and the indicated concentration of free Ca^{2+} (see the Experimental procedures section) in the presence (▲) or absence (△) of $40 \mu\text{M-GTP[S]}$. (b) Purified mast cells (2.5×10^4 /tube) were incubated for 5 min at 37 °C in mast-cell buffer containing $4.0 \mu\text{g}$ of digitonin/ml and either $10 \mu\text{M-Ca}^{2+}$ (▲) or 2.0 mM-EGTA (△), as well as the indicated concentration of GTP[S]. Histamine release was determined as described in the Experimental procedures section.

concentrations of the tumour-promoting phorbol ester TPA on Ca^{2+} - and GTP[S]-stimulated histamine release. The results of such an experiment are shown in Fig. 3. Exocytosis in response to the combination of GTP[S] ($40 \mu\text{M}$) and Ca^{2+} ($10 \mu\text{M}$) is only slightly enhanced by a 5 min pretreatment with up to 50 nM-TPA , since under these conditions the cells are nearly maximally stimulated. In contrast, preincubation of mast cells with $\geq 10 \text{ nM-TPA}$ increased histamine release in response to subsequent treatment with Ca^{2+} alone, from background levels to 55–60%. Exocytosis in response to $40 \mu\text{M-GTP[S]}$

Fig. 3. Effect of TPA on exocytosis from digitonin-permeabilized rat mast cells

Purified mast cells (2.5×10^4 /tube) were preincubated for 5 min at 37 °C with vehicle (0.1% DMSO) or the indicated concentration of TPA. The cells were then treated for an additional 5 min at 37 °C with mast-cell buffer containing 4 μg of digitonin/ml and 2.0 mM-EGTA (△), 10 μM- Ca^{2+} + 40 μM-GTP[S] (▲), 10 μM- Ca^{2+} alone (●) or 40 μM-GTP[S] alone (■). Histamine release was determined as described in the Experimental procedures section.

in the absence of Ca^{2+} increased from background levels without TPA to 60–70% with 20 nM-TPA. Incubation of cells with TPA followed by permeabilization in the absence of both Ca^{2+} and GTP[S] resulted in variable levels of histamine release (10–30% with 20 nM-TPA, $n = 3$ cell preparations; however, see also Tables 1 and 2). Control experiments (results not shown) demonstrated that the TPA analogues 4α-PDD and 4α-phorbol, neither of which activate PKC, had no effect on histamine release in the presence or absence of Ca^{2+} and GTP[S].

If, indeed, PKC sensitizes mast cells for exocytosis, one possibility is that activation of PKC might decrease the concentrations of Ca^{2+} and/or GTP[S] required for exocytosis. To examine this possibility, cells were pretreated with 25 nM-TPA or vehicle for 5 min at 37 °C, then permeabilized in the presence of the indicated second messenger molecules. Ca^{2+} thresholds of TPA-treated and untreated control cells were determined both with and without 40 μM-GTP[S] (Fig. 4). In the presence of GTP[S], a substantial level of exocytosis (60%) was achieved in TPA-pretreated cells in the absence of Ca^{2+} (2 mM-EGTA in the buffer). A reproducible increase in exocytosis (to approx. 90%) occurred as the Ca^{2+} concentration in the stimulus was increased to the micromolar range. Without TPA pretreatment, there was negligible histamine release unless both GTP[S] (40 μM) and Ca^{2+} (10 μM) were present. Without GTP[S] in the stimulus, histamine release from TPA-treated cells occurred at high concentrations of Ca^{2+} (10 μM). Thus in both the presence and the absence of GTP[S], TPA pretreatment reduced the Ca^{2+} requirement for exocytosis.

Fig. 4. Effect of TPA pretreatment on Ca^{2+} -dependence of exocytosis from permeabilized mast cells

Purified mast cells (2.5×10^4 /tube) were incubated for 5 min at 37 °C with either 25 nM-TPA (▲, △) or vehicle (■, □). Cells were then treated for an additional 5 min at 37 °C with mast-cell buffer containing 4.0 μg of digitonin/ml and the indicated concentrations of free Ca^{2+} in the presence (▲, ■) or absence (△, □) of 40 μM-GTP[S]. Histamine release was determined as described in the Experimental procedures section.

The effect of TPA on the GTP[S] requirement for exocytosis was investigated by challenging TPA-treated and untreated control cells with permeabilization buffers containing increasing concentrations of GTP[S], both with and without 10 μM- Ca^{2+} (Fig. 5). In agreement with

Fig. 5. Effect of TPA pretreatment on GTP[S]-dependence of exocytosis from permeabilized mast cells

Purified mast cells (2.5×10^4 /tube) were incubated for 5 min at 37 °C with 25 nM-TPA (▲, △) or vehicle (■, □). Cells were then treated with mast-cell buffer containing 4.0 μg of digitonin/ml and the indicated concentrations of GTP[S], as well as 10 μM-free Ca^{2+} (▲, ■) or 2.0 mM-EGTA (△, □). Histamine release was determined as described in the Experimental procedures section.

Table 1. Effect of staurosporine on exocytosis from permeabilized mast cells

Purified mast cells (2.5×10^4 /tube) were incubated for 5 min at 37 °C with 1.0 μ M-staurosporine or with vehicle (0.1% DMSO). Mast-cell buffer containing sufficient TPA to achieve a final concentration of 25 nM was added, and the cells were incubated for an additional 5 min at 37 °C. The exocytotic capability of the cells was assessed by adding mast-cell buffer containing sufficient digitonin, Ca^{2+} , GTP[S] and EGTA to achieve final concentrations of 4.0 μ g/ml, 10 μ M, 40 μ M and 2 mM respectively. Histamine release was determined as described in the Experimental procedures section. Values represent mean histamine release (%) $\pm$ S.D. from four independent experiments, each performed in duplicate.

Stimulus	Histamine release (%)	
	+ TPA	+ TPA + staurosporine
Ca^{2+} /GTP[S]	86 ± 7	81 ± 9
Ca^{2+}	49 ± 15	11 ± 3
GTP[S]	52 ± 10	11 ± 3
EGTA	25 ± 6	7 ± 5

the data presented in Figs. 3 and 4, 53% of cellular histamine was released from TPA-pretreated cells by 10 μ M- Ca^{2+} in the absence of GTP[S] (Fig. 5). This value increased steadily to approx. 85% as the GTP[S] concentration was increased to 100 μ M. Most striking, however, was the interaction between GTP[S] and TPA: preincubation with 25 nM-TPA increased exocytosis in response to 40 μ M-GTP[S] alone (2.0 mM-EGTA in the stimulus buffer) from 11% without TPA to 74% with TPA. Thus TPA pretreatment almost completely eliminated the Ca^{2+} requirement for exocytosis in digitonin-permeabilized mast cells. In this system, then, TPA has dual effects, reducing both the Ca^{2+} and the guanine nucleotide requirements for exocytosis.

Effects of staurosporine on TPA-stimulated exocytosis

Since phorbol ester treatment of mast cells might have diverse effects on cellular processes apart from activation of PKC, we addressed the question of PKC involvement in exocytosis using the PKC inhibitor staurosporine. This compound has been shown to inhibit PKC both *in vitro* and *in vivo* [36–39]; other kinases, such as myosin light-chain kinase and cyclic AMP-dependent protein kinase, are also inhibited by staurosporine, albeit at higher concentrations [36,37]. The results shown in Table 1 demonstrate that staurosporine pretreatment blocks the effects of TPA on the Ca^{2+} and GTP[S] requirements for exocytosis. Mast cells were pretreated with 1.0 μ M-staurosporine or with vehicle for 5 min at 37 °C before TPA treatment, permeabilization and challenge with Ca^{2+} and GTP[S]. Exocytosis in response to the combination of 25 nM-TPA, 10 μ M- Ca^{2+} and 40 μ M-GTP[S] was not affected by the staurosporine treatment. In contrast, exocytosis induced by combinations of both TPA and Ca^{2+} and TPA and GTP[S] was reduced to background levels by staurosporine treatment. The small amount of histamine release induced by TPA in the presence of EGTA was also abolished by staurosporine.

Fig. 6. Exocytosis from purified rat peritoneal mast cells as a function of staurosporine concentration

Purified mast cells (2.5×10^4 /tube) were incubated for 5 min at 37 °C with the indicated concentrations of staurosporine. Cells were then treated with 25 nM-TPA ($\blacktriangle$, $\blacksquare$) or with vehicle (0.05% DMSO; $\triangle$, $\square$) for an additional 5 min at 37 °C. Finally, the cells were incubated in mast-cell buffer containing 4.0 μ g of digitonin/ml and either 10 μ M- Ca^{2+} ($\blacktriangle$, $\triangle$) or 40 μ M-GTP[S] ($\blacksquare$, $\square$) for 5 min at 37 °C. Histamine release was determined as described in the Experimental procedures section.

Exocytosis induced by the combination of 40 μ M-GTP[S] and 10 μ M- Ca^{2+} in the absence of TPA was not affected by 1.0 μ M-staurosporine ($80 \pm 7\%$ release without staurosporine, $77 \pm 6\%$ release with 1.0 μ M-staurosporine; $n = 4$ cell preparations), indicating that in permeabilized cells, the exocytotic event itself is insensitive to the inhibitor. To further characterize the effects of staurosporine on TPA-stimulated exocytosis, experiments were performed to determine the concentration-dependence of inhibition. As shown in Fig. 6, half-maximal inhibition of both TPA/ Ca^{2+} -stimulated and TPA/GTP[S]-stimulated histamine release occurred at approx. 100 nM-staurosporine. This concentration is significantly higher than the concentration of staurosporine required for half-maximal inhibition of purified PKC ($K_i = 2.7$ nM [36]). However, it must be remembered that in intact cells the inhibitor must first gain access to the cytosol before inhibition can occur; thus the higher concentration of staurosporine required for inhibition of exocytosis in Fig. 6 might represent a simple permeability effect. In this regard it is noteworthy that our results are in agreement with inhibition studies in other cell types; for example, staurosporine in the micromolar range was required for complete inhibition of TPA-stimulated leukotriene D_4 receptor desensitization in RBL cells [38], and TPA-stimulated protein phosphorylation in human platelets [37].

Effect of PKC down-regulation on exocytosis in permeabilized cells

As an additional way of establishing whether the effects of TPA on exocytosis in the permeabilized cell system were due to the activation of PKC, we made use

Fig. 7. Down-regulation of mast-cell PKC by long-term phorbol ester treatment

Purified mast cells were incubated under the conditions described in Table 2 in the presence of various concentrations of TPA. Following incubation and washing, the cells were lysed in hot SDS/PAGE sample buffer and the lysates were analysed using Western blot techniques with anti-PKC antiserum as described in the Experimental procedures section. Lanes 1–5 contained cell lysates (1.5×10^6 cell equivalents/lane) from cells treated with 0, 2.5, 5, 10 and 25 nM-TPA for 20 h. Lane 6 contained a sample ($0.15 \mu\text{g}$) of partially purified rat brain PKC. The arrows (lanes 1 and 6) indicate the position of PKC.

of the down-regulation of PKC resulting from extended incubation with TPA. Such long-term phorbol ester treatment has been shown to result in down-regulation of PKC in a variety of cell types [40–42]. To assess PKC down-regulation in mast cells, cells were cultured in the presence of increasing concentrations of TPA for 20 h at 37°C and then solubilized in SDS/PAGE sample buffer, and the lysates were subjected to Western blot analysis using either polyclonal anti-PKC antiserum or non-immune IgY serum. Fig. 7 shows an autoradiogram from such an experiment. Lane 1 shows the lysate from mast cells cultured in the absence of TPA; lanes 2–5 show lysates from mast cells cultured in the presence of 2.5, 5, 10 and 25 nM-TPA respectively. Lane 6 shows a sample of partially purified rat brain PKC. An 80 kDa band comigrating with authentic rat brain PKC is visible in the control cells and is not detectable in cells treated with 2.5, 5, 10 or 25 nM-TPA. Control blots incubated with non-immune IgY failed to detect the 80 kDa band (results not shown). We conclude that PKC is down-regulated in mast cells treated with > 2.5 nM-TPA for 20 h at 37°C .

We next investigated the effects of TPA-mediated PKC down-regulation on exocytosis in the permeabilized cell system. Table 2 shows the results of such an experiment. In control cells incubated with 0.1% DMSO (vehicle) for 20 h at 37°C , exocytosis in response to Ca^{2+}

Table 2. Effect of long-term TPA treatment on exocytosis from purified rat peritoneal mast cells

Purified mast cells ($5 \times 10^5/\text{ml}$) were incubated for 20 h at 37°C in RPMI 1640 containing vehicle (0.05% DMSO; control) or 25 nM-TPA (PKC-depleted). Cells were washed and incubated either with vehicle (0.05% DMSO) or with 25 nM-TPA for 5 min at 37°C , and then permeabilized with mast-cell buffer containing $4.0 \mu\text{g}$ of digitonin/ml as well as $10 \mu\text{M}$ - Ca^{2+} + $40 \mu\text{M}$ -GTP[S], $10 \mu\text{M}$ - Ca^{2+} alone, $40 \mu\text{M}$ -GTP[S] alone, or 2 mM-EGTA, and incubated for an additional 5 min at 37°C . Histamine release was determined as described in the Experimental procedures section.

Stimulus	Histamine release (%)			
	Control cells		PKC-depleted cells	
	+ TPA	+ DMSO	+ TPA	+ DMSO
$\text{Ca}^{2+}/\text{GTP[S]}$	86 ± 7	79 ± 3	45 ± 3	40 ± 2
Ca^{2+}	31 ± 0	13 ± 5	9 ± 2	9 ± 3
GTP[S]	32 ± 0	9 ± 2	10 ± 0	9 ± 2
EGTA	13 ± 3	13 ± 5	10 ± 0	9 ± 2

and GTP[S] is unaffected (compare Table 2 and Fig. 3). Treatment of these control cells with TPA results in exocytosis upon challenge with either Ca^{2+} or GTP[S] alone. Cells in which PKC was down-regulated by TPA treatment, however, lost the ability to respond to subsequent TPA challenge (Table 2). Nevertheless, down-regulated cells still respond to the combination of Ca^{2+} and GTP[S], by releasing 40% of total cellular histamine (compared with 79% release from cells incubated with vehicle). Thus although the ability of down-regulated cells to respond to TPA is completely abrogated, such cells are still capable of mounting a substantial response to Ca^{2+} and GTP[S]. These results support the proposal that PKC modulates, but is not required for, exocytosis in digitonin-permeabilized rat mast cells.

DISCUSSION

The cross-linking of cell surface IgE receptors on mast cells initiates a cascade of biochemical events culminating in the exocytotic release of histamine and other inflammatory mediators. An early post-receptor event is the activation of PLC by a GTP-binding protein (G_P) [19,23,24]. Generation of IP_3 and DAG by PLC-catalysed hydrolysis of PIP_2 results in the liberation of Ca^{2+} from intracellular stores and the activation of PKC respectively. While elevated intracellular Ca^{2+} is a hallmark of regulated exocytosis, the precise role of PKC in stimulus–secretion coupling is currently unclear. Nevertheless, IgE receptor cross-linking is known to stimulate translocation of PKC to the plasma membrane (site of DAG production [41]), and TPA, a stable DAG analogue that activates PKC, has been shown to increase the sensitivity of intact mast cells to low levels of the Ca^{2+} ionophore A23187 [27,28]. These results, together with a large body of related work in other cell types (see [21] for a review), suggest that PKC may modulate the exocytotic release of histamine from mast cells.

As a first step in our investigation of the potential

involvement of PKC in mast cell exocytosis, we tested the effect of TPA pretreatment on the threshold concentrations of Ca^{2+} and GTP[S] required for exocytosis in a digitonin-permeabilized mast cell system. Whereas digitonin has been used to permeabilize many secretory cell types, including mast cells [43–45], digitonin-permeabilized rat peritoneal mast cells have not been extensively studied; however, exocytosis in mast cells permeabilized with the bacterial toxin SLO has been the subject of recent investigation [4,6,7]. Exocytosis from digitonin-permeabilized mast cells requires the addition of both Ca^{2+} and GTP[S]. The threshold concentrations of these substances required for half-maximal histamine release are $1.3 \mu\text{M}$ for Ca^{2+} and $5 \mu\text{M}$ for GTP[S]. These values are similar to those obtained by Gomperts and his colleagues with SLO-permeabilized mast cells [4,6,7]. The concentration-dependence for Ca^{2+} in the digitonin-permeabilized mast cells is also in agreement with results of permeabilization studies conducted with a variety of other cell types, including neutrophils [15], HL60 cells [5], platelets [2,16], adrenal chromaffin cells [1,8,11–13,17] and PC12 cells [9,14]. This threshold Ca^{2+} concentration required for exocytosis in all of these permeabilized cell systems is nearly equivalent to the peak Ca^{2+} concentrations measured *in vivo* in RBL cells [46].

The observation by Cockcroft *et al.* [6] that exocytosis stimulated by the combination of GTP[S] and Ca^{2+} occurs even in the absence of PLC activation suggests that a second GTP-reactive site, probably a second GTP-binding protein (G_E), regulates exocytosis. Guanine-nucleotide-dependent, Ca^{2+} -independent exocytosis from RINm5F cells [3] is also consistent with a role for G_E . Given these results, we believe it likely that the dual requirement for both Ca^{2+} and GTP[S] in our digitonin-permeabilized mast cell system is a reflection of G_E activation.

Regarding the effects of TPA on mast cell exocytosis, we established that pretreatment of cells with TPA reduces the concentrations of both Ca^{2+} and GTP[S] required for exocytosis from permeabilized cells. As shown in Figs. 5 and 6, cells pretreated with TPA were able to release nearly all of their histamine when challenged with GTP[S] alone. This result could be interpreted to mean that TPA-treated cells require no Ca^{2+} for exocytosis. However, it must be kept in mind that in our experiments, as in those of Gomperts and his colleagues, the stimuli (Ca^{2+} and GTP[S]) are administered in the permeabilization buffer. At the instant of permeabilization, cells contain resting levels of Ca^{2+} and nucleotides. Under these conditions, it is possible that low concentrations of Ca^{2+} may persist in the vicinity of the secretory vesicles, in spite of the inclusion of 2 mM-EGTA in the permeabilization buffer. Thus, although TPA pretreatment reduces the Ca^{2+} requirement for exocytosis, it is not clear whether the need for Ca^{2+} is entirely eliminated. Nevertheless, our results are totally consistent with the observation that antigen can stimulate significant histamine release from TPA-treated RBL cells under conditions where there is no detectable increase in cytosolic Ca^{2+} concentration [25].

With respect to the GTP[S] requirement for exocytosis from permeabilized mast cells, it should be emphasized that GTP[S] is a non-physiological analogue of GTP. Thus whereas resting cells contain no GTP[S], it is

probable that under some circumstances (TPA treatment?), the low levels of cellular GTP present in permeabilized cells may be able to sustain some exocytosis, even in the absence of exogenously added GTP[S]. This may explain why TPA-treated cells can release up to 50% of their histamine when stimulated with $10 \mu\text{M}$ - Ca^{2+} in the absence of GTP[S] (Figs. 5 and 6), a result consistent with the observation that the sensitivity of both mast cells [27,28] and RBL cells [25] to ionophore alone (in the absence of antigen) is increased by pretreatment with TPA.

It should also be noted that TPA pretreatment reproducibly resulted in a small and somewhat variable (10–20% above background) concentration-dependent release of histamine from intact mast cells (i.e. without permeabilization). Though this increase in background release in TPA-treated cells may be non-specific, the observation that it can be inhibited by staurosporine (Table 1) suggests that TPA treatment may actually activate a small percentage of resting cells.

Although PKC appears to be the intracellular target of TPA [47], a positive effect of TPA on exocytosis should not be used uncritically to implicate PKC in exocytosis. With this in mind, we examined the effects of inhibition or down-regulation of PKC on exocytosis. Inhibition of PKC with staurosporine and down-regulation via prolonged incubation with TPA differ fundamentally with respect to the mechanism by which they reduce PKC activity, yet both completely blocked exocytosis in response to TPA/ Ca^{2+} and TPA/GTP[S] (Fig. 6, Tables 1 and 2). These results strongly suggest that PKC is responsible for the observed TPA-induced increase in the sensitivity of exocytosis to Ca^{2+} and GTP[S] documented in Figs. 3–5.

We have not formally eliminated the possibility that there is residual PKC in the down-regulated cells, below the limit of detection of the Western blot assay. However, it is clear that the down-regulated cells lost all ability to respond to TPA. Nevertheless, they were still able to mount a substantial exocytotic response to the combination of Ca^{2+} and GTP[S]. This finding, in conjunction with the maintenance of exocytotic activity in response to Ca^{2+} and GTP[S] in cells treated with high concentrations of the known PKC inhibitor staurosporine, support our contention that activation of PKC is not required for exocytosis in the permeabilized cell system. This conclusion is consistent with the observation that down-regulated RBL cells were able to mount a small exocytotic response to antigen [41].

In summary, we have used a digitonin-permeabilized rat mast cell system to study inter-relationships between Ca^{2+} , guanine nucleotides and the phorbol ester TPA in the signal transduction pathways leading to exocytosis. In the absence of TPA, both Ca^{2+} and the stable GTP analogue GTP[S] are required for the stimulation of exocytosis. Pretreatment with TPA results in a concentration-dependent enhancement of exocytosis in response to either Ca^{2+} or GTP[S] alone. This enhancement may be blocked by inhibition or by down-regulation of PKC. The results suggest that PKC-catalysed phosphorylation of some element(s) of the exocytotic machinery sensitizes mast cells for exocytosis. Both the identity of the phosphorylated protein(s) that mediates PKC modulation and the specific mechanism by which it reduces the Ca^{2+} and guanine nucleotide requirements for exocytosis remain to be determined.

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