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Inhibition of Cromolyn-Induced Phosphorylation of a 78-kDa Protein by Phorbol Esters in Rat Peritoneal Mast Cells

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ABSTRACT. Disodium cromoglycate (cromolyn) is a well documented inhibitor of immunologically-induced histamine release from rat peritoneal mast cells and has been shown to stimulate the phosphorylation of a mast cell protein of apparent molecular mass 78,000 Da (78 kDa), an event which may be involved in terminating secretion. Here we aimed to determine the role of the ubiquitous enzyme, protein kinase C, in the phosphorylating activity of cromolyn by examining the effects of phorbol esters (activators of protein kinase C) on protein phosphorylation in [^{32}P]orthophosphate loaded rat peritoneal mast cells. Protein kinase C-activating phorbol esters such as 12-O-tetradecanoyl phorbol-13-acetate (TPA) and 4β-phorbol 12,13-dibutyrate (PdBu) were found to potently inhibit cromolyn-induced phosphorylation when added to mast cells simultaneously with cromolyn (IC_{50} 22 and 79 nM respectively). 4α-Phorbol 12,13-didecanoate (PdD), a phorbol ester which does not activate protein kinase C, had no effect on cromolyn-induced phosphorylation. Addition of TPA to mast cells previously exposed to cromolyn for 60 sec (i.e. when 78-kDa protein phosphorylation is maximal) also caused a very rapid dephosphorylation of the 78-kDa protein. Phosphorylation of the 78-kDa protein can also be induced by dibutyryl cyclic GMP and this action was similarly inhibited by TPA and PdBu. Cromolyn inhibited secretion induced by anti-IgE, but not by TPA, and thus inhibition of secretion by cromolyn is further correlated to its phosphorylation of the 78-kDa protein. The data suggest that the inhibitory action of cromolyn on mast cell secretion and phosphorylation of the 78-kDa protein are not mediated through a phorbol ester-sensitive protein kinase C, but more likely that such an enzyme could be involved in regulating dephosphorylation of the 78-kDa protein. Further explanations for this novel dephosphorylating activity of phorbol esters are discussed. BIOCHEM PHARMACOL 55;5:585–594, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cromolyn; phorbol esters; mast cells; protein phosphorylation; protein kinase C; secretion

The primary effector cell in allergic reactions is the mast cell. These cells express on their surface a receptor protein, FcεRI, which binds with high affinity the Fc region of immunoglobulin E (IgE)‡ [1]. Aggregation of surface IgE molecules by multivalent antigen results in the activation of an array of signal transduction pathways leading eventually to the release of inflammatory mediators from mast cell secretory granules [2, 3]. These mediators include preformed substances such as histamine, proteases and cytokines, and newly synthesized mediators such as leukotrienes, prostaglandins and platelet activating factor [4].

The pathways leading from FcεRI aggregation to mediator release are as yet poorly elucidated, although many signalling mechanisms have been implicated from studies with mast cells and various secretory cell lines such as RBL-2H3 cells. These include; an elevation of intracellular Ca^{2+} through an influx of extracellular Ca^{2+} or mobilization of intracellular stores [5–7], elevation of cyclic nucleotides [8], activation of G-proteins [9–11], activation of phospholipase A_2 yielding arachidonic acid and eicosinoid metabolites [12], tyrosine kinase activation [13] and the activation of phospholipase C [11] and protein kinase C (PKC) [6, 14, 15]. Understanding the importance and interaction of these complex signals in initiating a secretory response has been the goal of a wealth of research effort in recent decades.

Other biochemical events seen after immunological or non-immunological challenge of mast cells include the rapid (within 15 sec) phosphorylation of three proteins of apparent molecular mass 42, 59 and 68 kDa [16–18]. The 59-kDa species has recently been identified as vimentin, a protein found in intermediate filaments [19]. The time course of phosphorylation of these three proteins roughly corresponds to the onset of secretion, therefore it has been assumed that these proteins are involved in the activation of exocytosis. The phosphorylation of a fourth protein of

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‡ *Abbreviations:* cromolyn, disodium cromoglycate; DBcGMP, N^2,2′-O-dibutyrylguanosine 3′:5′-cyclic monophosphate; IgE, immunoglobulin E; PdBu, 4β-phorbol 12, 13-dibutyrate; PdD, 4α-phorbol 12, 13-didecanoate; PKC, protein kinase C; THG, HEPES buffered Tyrode containing gelatin (0.1% w/v); TPA, 12-O-tetradecanoyl phorbol-13-acetate.

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apparent molecular mass 78 kDa is observed after about 2 min of challenge [16–18]. This phosphorylation is assumed to be part of a mechanism by which secretion is terminated, especially in the light of evidence that this phosphorylation can be specifically stimulated by agents such as dibutyryl cyclic GMP (DBcGMP), disodium cromoglycate (cromolyn) and the cromolyn analogue, nedocromil, all of which inhibit secretion [18, 20, 21].

Cromolyn has been successfully used as a prophylactic treatment for allergic bronchial asthma for more than twenty years. Despite intensive research the mode of action of cromolyn in the treatment of asthma remains to be fully understood. It is possible that the efficacy of cromolyn lies in its ability to inhibit the secretion of potent chemical mediators from lung mast cells [22]. In rat peritoneal mast cells (the most commonly used animal model) cromolyn inhibits histamine release evoked by immunological (via FcεRI) and non-immunological stimuli (e.g. compound 48/80) [23, 24]. The mechanism by which cromolyn inhibits mast cell secretion is unclear. One possibility is that cromolyn causes inhibition of the cell membrane calcium gating mechanism which operates upon immunological stimulation [25]. A second proposal (see above) is that the cromolyn-stimulated phosphorylation of a mast cell protein of apparent molecular mass 78-kDa forms part of a signalling mechanism involved in terminating secretion [18, 20]. The dose responses for stimulation of 78-kDa protein phosphorylation correlate with those for inhibition of histamine release. Furthermore, both 78-kDa protein phosphorylation and inhibition of histamine release displayed tachyphylaxis [20], adding evidence for a causal relationship between the two. It has also been postulated that cromolyn exerts its inhibitory effect on secretion by interacting with PKC [26]. The experiments described here aimed to determine the role, if any, of PKC in the cromolyn-induced phosphorylation of the 78-kDa protein.

MATERIALS AND METHODS

Disodium cromoglycate (cromolyn) was kindly provided by Fisons plc. Loughborough and Ascitic fluid from Lou/F rats bearing the IgE-secreting IR 162 immunocytoma was a generous gift from Dr. E. Blair of the Department of Biochemistry and Molecular Biology, University of Leeds. 12-O-tetradecanoyl phorbol-13-acetate, 4β-phorbol 12, 13-dibutyrate and 4α-phorbol 12, 13-didecanoate were purchased from Sigma Chemical Company Ltd. [^{32}P]orthophosphate and S-adenosyl-L-[methyl-^{3}H]methionine were purchased from Amersham International plc. and sheep anti-rat IgE serum (lyophilized) was obtained from Miles Laboratories Ltd.

The buffer used throughout was a Tyrode, HEPES and gelatin buffer (THG) of the following composition: NaCl 137 mM, KCl 2.7 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, glucose 5.6 mM, gelatin 0.1% (w/v), HEPES 10 mM and adjusted to pH 7.4. Phorbol esters were dissolved in DMSO at a concentration of 5 mg/mL and then serially diluted in THG to the required concentration.

Purification of Rat Peritoneal Mast Cells

Rat peritoneal mast cells were purified on a Percoll gradient as previously described [18]. Briefly, male Sprague–Dawley rats (250–900 g) were killed by an atmosphere of carbon dioxide and the peritoneal cavity lavaged with 20–40 mL ice-cold THG containing heparin (5 units/mL). Washings were pooled and centrifuged (200 × *g*, 5 min, 4°) and the cells resuspended in 1.05 mL THG. Precisely 1.0 mL of cell suspension was mixed with 4.0 mL 90% Percoll in isoosmotic THG (comprising 3.6 mL Percoll and 0.4 mL 10-fold concentrated THG) to give a final density of 1.106 g/mL. The Percoll/cell suspension was overlayed with 1.0 mL THG and then centrifuged (200 × *g*, 15 min, 4°). The resulting supernatant was discarded and the mast cell pellet washed, to remove residual Percoll, by resuspension in approximately 10 mL THG followed by centrifugation (200 × *g*, 5 min, 4°). Mast cells were routinely obtained at >95% purity as assessed by differential staining with Kimura's stain [27] and with a yield of approximately 10^6 cells per rat.

Loading of Mast Cells with [^{32}P]orthophosphate and Preparation of Samples for SDS-Polyacrylamide Gel Electrophoresis

Mast cells in 0.2–0.3 mL THG and at a density of 0.1 × 10^7–2.0 × 10^7 cells/mL were incubated with [^{32}P]orthophosphate (final concentration 0.1–1.0 mCi/mL) for 30 min at 37°. Cells were washed twice, to remove excess [^{32}P]orthophosphate, by centrifugation in 4 mL THG and then resuspended to a final density of 0.5 × 10^6–7.0 × 10^6 cells/mL. Appropriate volumes of the labelled cell suspension were placed in 1.5 mL microcentrifuge tubes and stored on ice for as short a period as possible (generally a few to 30 min) prior to use. Before addition of test compound or buffer, cell suspensions were incubated at 37° for 2 min. One vol. of test compound or buffer (also pre-warmed to 37°) was then added to three vol. of cell suspension. Incubation at 37° was continued for the required period and the cells lysed by the addition of one vol. of a 5-fold concentrated SDS-sample buffer (see below). Tubes were immediately transferred to a boiling water bath for 3 min, allowed to cool to room temperature and then centrifuged (12,000 × *g*, 2 min) in a microcentrifuge prior to loading samples on electrophoresis gels. Routinely, experiments were repeated on one or more occasions and these yielded similar results to those shown.

SDS-Polyacrylamide Gel Electrophoresis and Autoradiography

SDS-polyacrylamide gradient slab gels (0.75 mm thickness) were made according to the discontinuous buffer system

described by Laemmli [28]. Resolving gels comprised linear acrylamide gradients of 7.5–15% (w/v) and were overlayed with a 3.75% (w/v) acrylamide stacking gel. A 5-fold concentrated SDS-sample buffer used to prepare samples had the following composition: 10% (w/v) SDS, 0.33 M Tris-HCl pH 6.8, 0.75 M sucrose, 10% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue. Gels were calibrated by use of the SDS-7 and SDS-6H molecular mass marker kits produced by Sigma and also including human transferrin (80 kDa). Electrophoresis was carried out at 70 V constant voltage overnight until the bromophenol blue tracking dye just left the gel. Gels were stained with PAGE Blue 83 (BDH Chemicals Ltd), destained and dried. Direct autoradiography was performed at room temperature using Kodak X-Omat RP film or Fuji RX film typically for 2–5 days.

For the quantitation of autoradiograph band intensities an LKB Ultrascan XL laser densitometer was employed. Because of a variable background on autoradiographs, peak heights were assessed by manual measurements made on the densitometer traces. Stained gels were also scanned and the peak height of the major protein band (27 kDa) determined. To correct for slight differences in the amount of protein on each lane, peak height values of autoradiograph bands were divided by the peak height value of the stained 27-kDa band from the corresponding lane of the gel. The values obtained were then normalised relative to the value obtained for buffer-treated cells at the earliest time point employed.

Histamine Release from Rat Peritoneal Mast Cells

Mast cells in THG buffer were first sensitized with IgE by incubation with a 1/10 dilution of Ascites E-myeloma for 1 hr at 37°. After washing twice in THG, cells were resuspended to 10^5 cells/mL, prewarmed to 37° and 0.1 mL aliquots added to 0.1 mL of challenge at 37°. The release process was arrested after 12 min by the addition of 0.8 mL ice-cold THG, the tubes centrifuged briefly in a microcentrifuge and 0.5 mL of each supernatant retained for assay. The total histamine content of cells was determined on 0.1 mL aliquots of cells lysed with 0.9 mL H_2O and treated for 5 min in a bath sonicator. Histamine was determined by an enzymatic isotopic assay essentially as previously described [29] using S-adenosyl-L-[methyl-^{3}H]methionine (500 mCi/mmol) and histamine-*N*-methyl transferase prepared from rat kidney.

RESULTS

Initial experiments examined the effects of cromolyn and the phorbol ester, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), a well characterised activator of PKC, on mast cell protein phosphorylation. Figure 1a shows that in unstimulated cells a variety of proteins are phosphorylated. A protein of apparent molecular mass 78,000 Da (78 kDa) is seen to be weakly phosphorylated in unstimulated cells, but exposure to cromolyn (100 μM) caused about a 5-fold increase in the labelling of this band. TPA (150 nM) did not stimulate phosphorylation of the 78-kDa protein, but, surprisingly, caused a decrease in the labelling of this band to below the level seen in the presence of buffer alone (Fig. 1b). Simultaneous addition of TPA with cromolyn almost completely blocked cromolyn-induced phosphorylation of the 78-kDa protein. Indeed, when cromolyn and TPA were added together, phosphorylation of the 78-kDa protein was similar to the control level after 30 sec and thereafter fell to below the control level, as observed with TPA alone. With cromolyn alone maximal phosphorylation of the 78-kDa protein occurred after 30–60 sec and had only slightly decreased by 3 min. When TPA was added to mast cells 60 sec after addition of cromolyn (i.e. when phosphorylation of the 78-kDa protein is maximal) the 78-kDa protein was rapidly dephosphorylated, again to below the control level. Figure 2 shows that at all time points tested (i.e. 30–180 sec) TPA increased the incorporation of radioactive phosphate into two proteins of apparent molecular mass 46 and 64 kDa and these changes were unaffected by the presence of cromolyn.

To further investigate the mechanism of the inhibitory effect of TPA on cromolyn-induced phosphorylation two other phorbol esters were used in the phosphorylation assay. These were 4β-phorbol 12,13-dibutyrate (PdBu) that, like TPA, is also an activator of PKC, and 4α-phorbol 12,13-didecanoate (PdD) that does not activate PKC [30, 31]. Figure 3 shows that TPA and PdBu had identical effects on cromolyn-induced phosphorylation of the 78-kDa protein in that a small increase in labelling was evident after 30 sec that thereafter fell to below the control level. In contrast, cromolyn-stimulated phosphorylation of the 78-kDa protein was unaffected by the simultaneous addition of PdD. PdBu, like TPA, also stimulated phosphorylation of the 46- and 64-kDa proteins whereas PdD was without effect. Figure 4 shows that addition of TPA or PdBu to mast cells 1 min before cromolyn also completely blocked cromolyn-induced phosphorylation whereas PdD again had no effect. Addition of phorbol ester 10 min prior to cromolyn gave similar results (data not shown).

The potencies of TPA and PdBu in the antagonism of cromolyn-induced phosphorylation were compared in a dose response experiment using simultaneous addition of phorbol ester with a fixed concentration of cromolyn (100 μM). All incubations were arrested after 60 sec and the resulting autoradiograph is shown in Fig. 5a. TPA and PdBu produced similar inhibition curves for their effects on cromolyn-induced phosphorylation (Fig. 5b). Apparent IC_{50} values were 22 and 79 nM for TPA and PdBu respectively. Maximal inhibition of 78-kDa protein phosphorylation, which was virtually complete, occurred at approximately 0.3–1.0 μM for both agents. Phosphorylation of the 46-kDa protein was seen to increase gradually with increasing concentration of both phorbol esters until a maximum level was reached at about 30 nM (Fig. 5c). Phorbol ester-stimulated phosphorylation of the 64-kDa band followed a

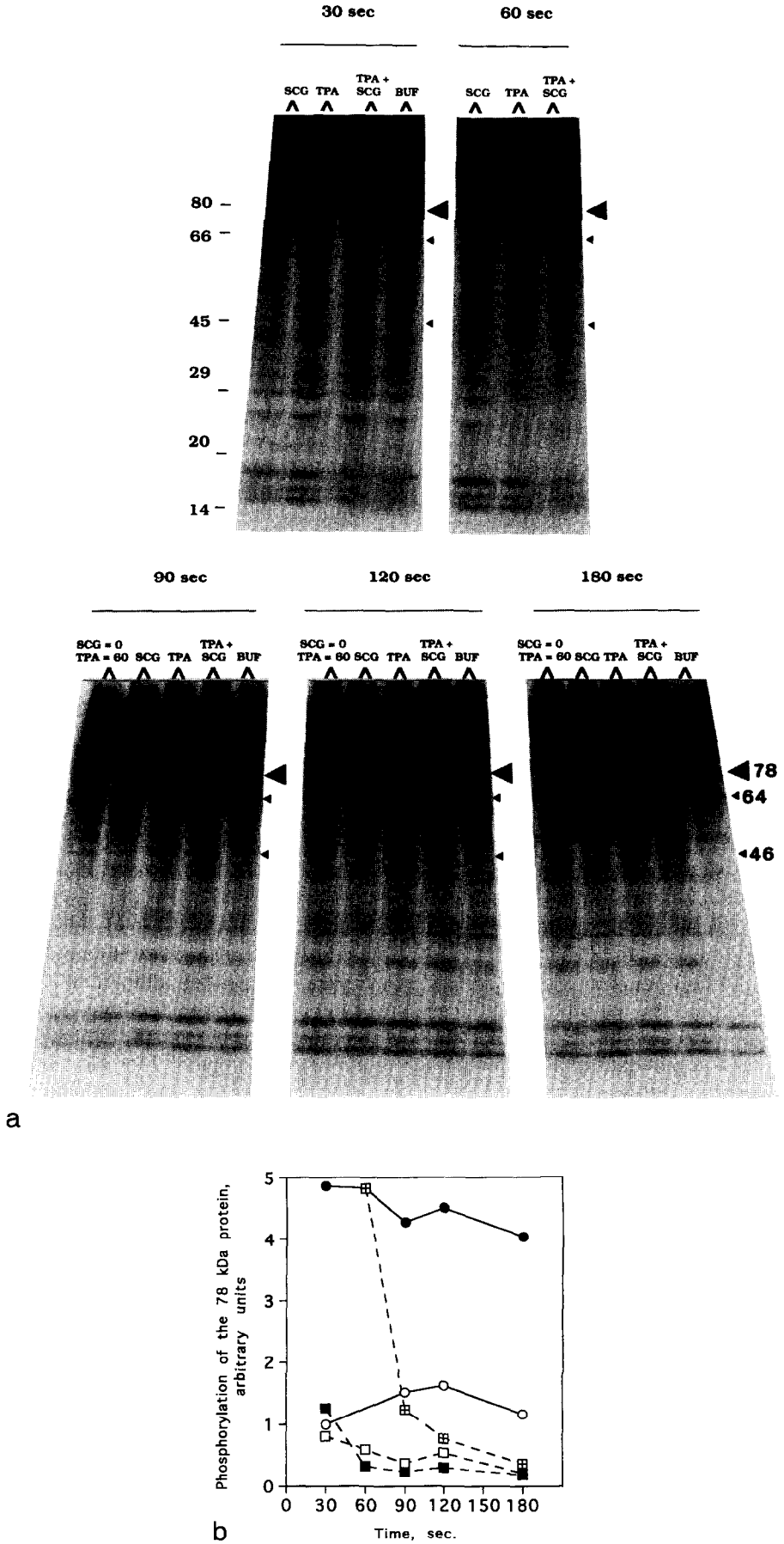
30 sec
60 sec
SCG
TPA
TPA + SCG
BUF
80
66
45
29
20
14
90 sec
120 sec
180 sec
SCG = 0 TPA = 60
78
64
46
a
Phosphorylation of the 78 kDa protein, arbitrary units
0
1
2
3
4
5
0 30 60 90 120 150 180
Time, sec.
b

FIG. 1. 78-kDa protein phosphorylation following the addition of TPA simultaneously with, and one minute after, the addition of cromolyn to rat mast cells. (a) Autoradiograph. Rat mast cells were labelled with [^{32}P]orthophosphate as described in the methods section and the phosphorylation assay performed using the following stimuli: SCG = O, TPA = 60: cromolyn (100 μM, final concentration) added at 0 sec, and TPA (150 nM, final concentration) added at 60 sec, SCG: cromolyn (100 μM) added at 0 sec, TPA: TPA (150 nM) added at 0 sec, TPA + SCG: TPA (150 nM) and cromolyn (100 μM) added simultaneously at 0 sec, BUF: THG buffer added at 0 sec. Samples were removed and added to SDS-sample buffer at the times shown, analysed by SDS-PAGE and the resulting autoradiograph is shown. Figures on the left indicate molecular mass markers in kDa. Large arrowheads indicate the position of the 78-kDa phosphoprotein and small arrowheads indicate the 64- and 46-kDa phosphoproteins. (b) Phosphorylation of the 78-kDa protein as assessed by densitometer scanning of the autoradiograph shown in (a), cromolyn (●), THG buffer (○), TPA (□), cromolyn + TPA added simultaneously (■); cromolyn added at 0 sec followed by TPA added at 60 sec (⊞).

less clear pattern and demonstrated only a crude relationship to phorbol ester concentration. Quantifying changes in the labelling of the 64-kDa band is inherently problematic because it is strongly labelled in unstimulated cells and the relative increase induced by the phorbol esters is small.

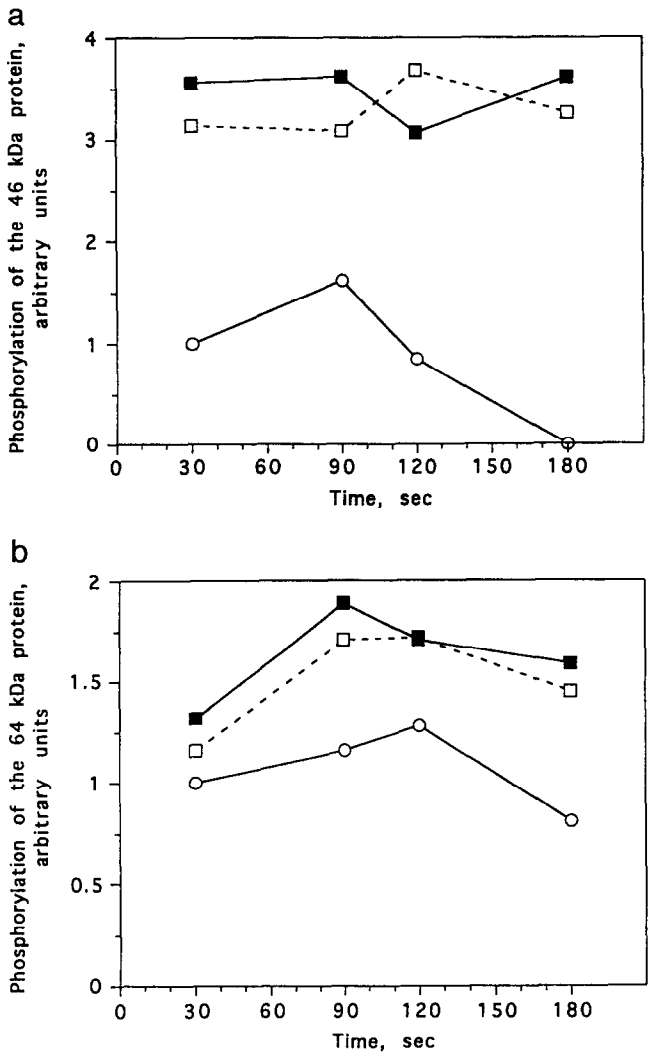

FIG. 2. TPA-induced phosphorylation of the 46- and 64-kDa proteins in rat mast cells in the presence and absence of cromolyn. This figure shows further data from the experiment described in Fig. 1 and depicts the extent of phosphorylation of the 46-kDa (Fig. 2a) and 64-kDa (Fig. 2b) proteins as assessed by densitometer scanning. THG buffer (○), 150 nM TPA (□), 150 nM TPA + 100 μM cromolyn (■). Values for THG buffer at 30 sec were arbitrarily given the value 1.0.

A previous study has demonstrated that phosphorylation of the 78-kDa protein is also stimulated by dibutyryl cyclic GMP (DBcGMP) [18]. Figure 6 clearly confirms this observation and shows that simultaneous addition of TPA to mast cells also blocks DBcGMP-induced phosphorylation of the 78-kDa protein. Pre-incubation of mast cells with TPA for 10 min also completely blocked DBcGMP-induced phosphorylation.

We also determined the effect of cromolyn on histamine release induced by anti-IgE and TPA. Figure 7 shows that while anti-IgE and TPA evoked similar levels of release, anti-IgE-induced release was inhibited 66% by 100 μM cromolyn, whereas TPA-induced release was unaffected. Furthermore, addition of anti-IgE simultaneously with cromolyn to [^{32}P]orthophosphate-labeled mast cells did not affect cromolyn-induced phosphorylation of the 78-kDa protein (data not shown).

DISCUSSION

In this paper we report that the phorbol esters, TPA and PdBu, potently inhibit cromolyn-induced phosphorylation of a 78-kDa protein in rat mast cells, an event postulated to be involved in terminating secretion and to mediate the inhibitory effect of cromolyn on secretion. Cromolyn failed to inhibit histamine release evoked by TPA, conditions under which cromolyn-induced phosphorylation of the 78-kDa protein is greatly reduced. We also found that anti-IgE, a secretory stimulus which is potently inhibited by cromolyn, did not affect the phosphorylation of the 78-kDa protein induced by cromolyn. These findings further support the link between the phosphorylation of the 78-kDa protein and cromolyn inhibition of histamine release and suggest that phorbol ester-sensitive PKC is not responsible for this phosphorylation event.

Several studies have examined the effects of phorbol esters on protein phosphorylation in rat mast cells [32–35]. These differ significantly as to which proteins undergo altered phosphorylation and in the relative changes that occur when compared to control samples. In our hands, TPA induced the phosphorylation of proteins of approximate molecular mass 64 and 46 kDa. On occasion, we also observed phorbol ester-stimulated phosphorylation of proteins of molecular mass 75, 69 and 60 kDa (Figs. 4 and 5a). The most pronounced and consistent increase was in the 46-kDa protein and at least two other reports indicate the

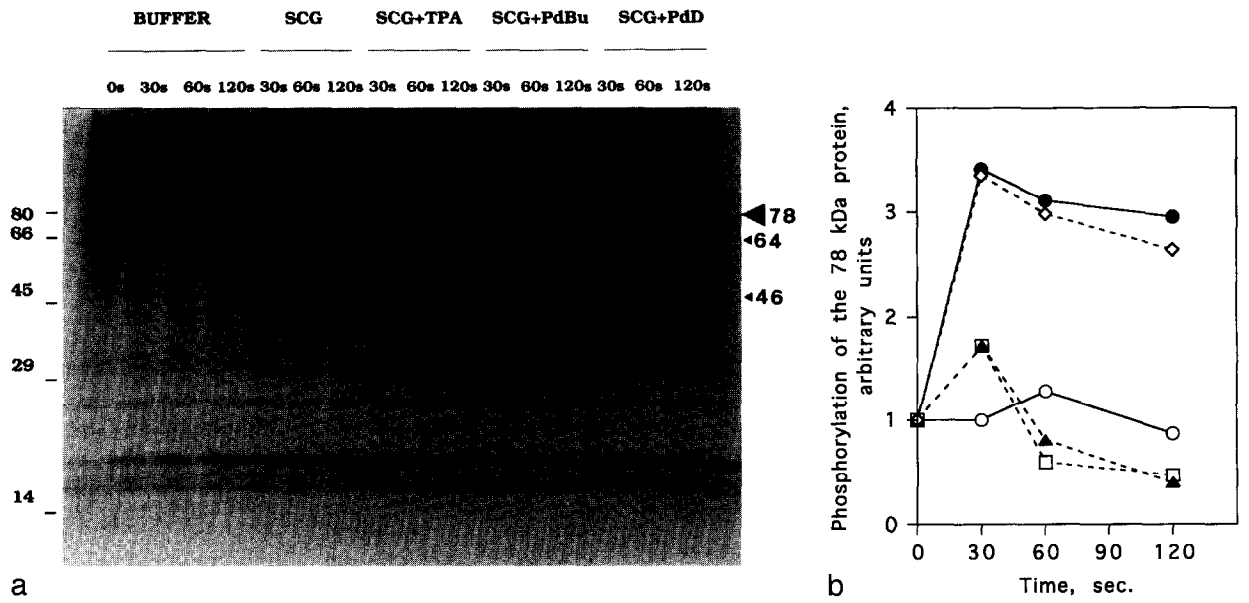

FIG. 3. The effects of TPA, PdBu and PdD on protein phosphorylation in rat mast cells, in the presence of cromolyn. (a) Autoradiograph. Rat mast cells were labelled with [^{32}P]orthophosphate and the phosphorylation assay performed as described. TPA, PdBu and PdD were used at a final concentration of 150 nM and were added simultaneously with cromolyn (100 μM, final concentration). Aliquots were removed at the times shown and analysed by SDS-PAGE. The resulting autoradiograph is shown. Figures on the left indicate molecular mass markers in kDa. The 78-, 64- and 46-kDa bands are indicated with arrows on the right. (b) Phosphorylation of the 78-kDa protein as assessed by densitometer scanning of the autoradiograph shown in (a). cromolyn (●), THG buffer (○), cromolyn + TPA (□), cromolyn + PdBu (▲), cromolyn + PdD (◇). The value for THG buffer at 0 sec was arbitrarily given the value 1.0.

greatest change to be in proteins of similar molecular mass [33, 35]. It seems likely that the increased phosphorylation of mast cell proteins caused by TPA and PdBu is due to the direct activation of a phorbol ester-sensitive PKC. Since cromolyn neither interfered with phorbol ester-induced phosphorylation of these two proteins, nor itself induced this phosphorylation, it seems likely that cromolyn does not inhibit PKC activation by phorbol esters, nor does it activate the enzyme either directly or indirectly. These data, taken together with the finding that the drug does not enter the cell to exert its effects [36], do not support the proposal that cromolyn interacts directly with PKC [26].

It is also clear from this research that phorbol ester-sensitive PKC is not directly responsible for phosphorylating the 78-kDa protein since PKC-activating phorbol esters are not stimulatory. A previous report has demonstrated that the protein kinase inhibitors, H8 and H89 (100 μM), did not inhibit cromolyn-induced phosphorylation [37]. This finding also argues against a role for PKC in cromolyn-induced phosphorylation since PKC inhibition would be expected with these agents at the concentrations employed [38, 39]. However, it is conceivable that a phorbol ester-sensitive PKC may play a role in regulating the phosphorylation status of the 78-kDa protein by an indirect mechanism. The concentrations at which TPA and PdBu are effective against cromolyn-induced phosphorylation, and their relative potencies, are consistent with their effects being mediated through PKC. If this is the case, phorbol ester activation of PKC must lead to the inhibition of another kinase (e.g. the kinase activated by cromolyn), or

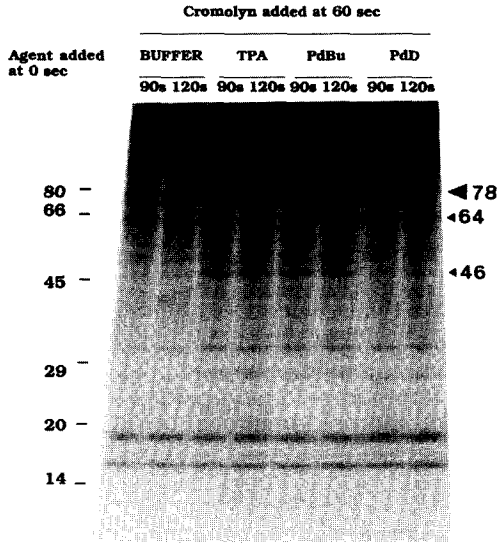

FIG. 4. The effects of TPA, PdBu and PdD on protein phosphorylation in rat mast cells when added 1 min before cromolyn. Autoradiograph showing the effects of TPA, PdBu and PdD on 78-kDa protein phosphorylation when added 60 sec before cromolyn. Rat peritoneal mast cells were labelled with [^{32}P]orthophosphate and the phosphorylation assay performed as described. Buffer, TPA, PdBu or PdD were added 60 sec prior to cromolyn (100 μM, final concentration) and aliquots removed after a further 30 and 60 sec (labelled in figure as 90 and 120 sec from first addition) and added to SDS-sample buffer. Phorbol esters were added to a final concentration of 200 nM, but on subsequent addition of cromolyn a concentration of 150 nM was achieved. Figures on the left indicate molecular mass markers in kDa. The 78-, 64- and 46-kDa bands are indicated with arrows on the right.

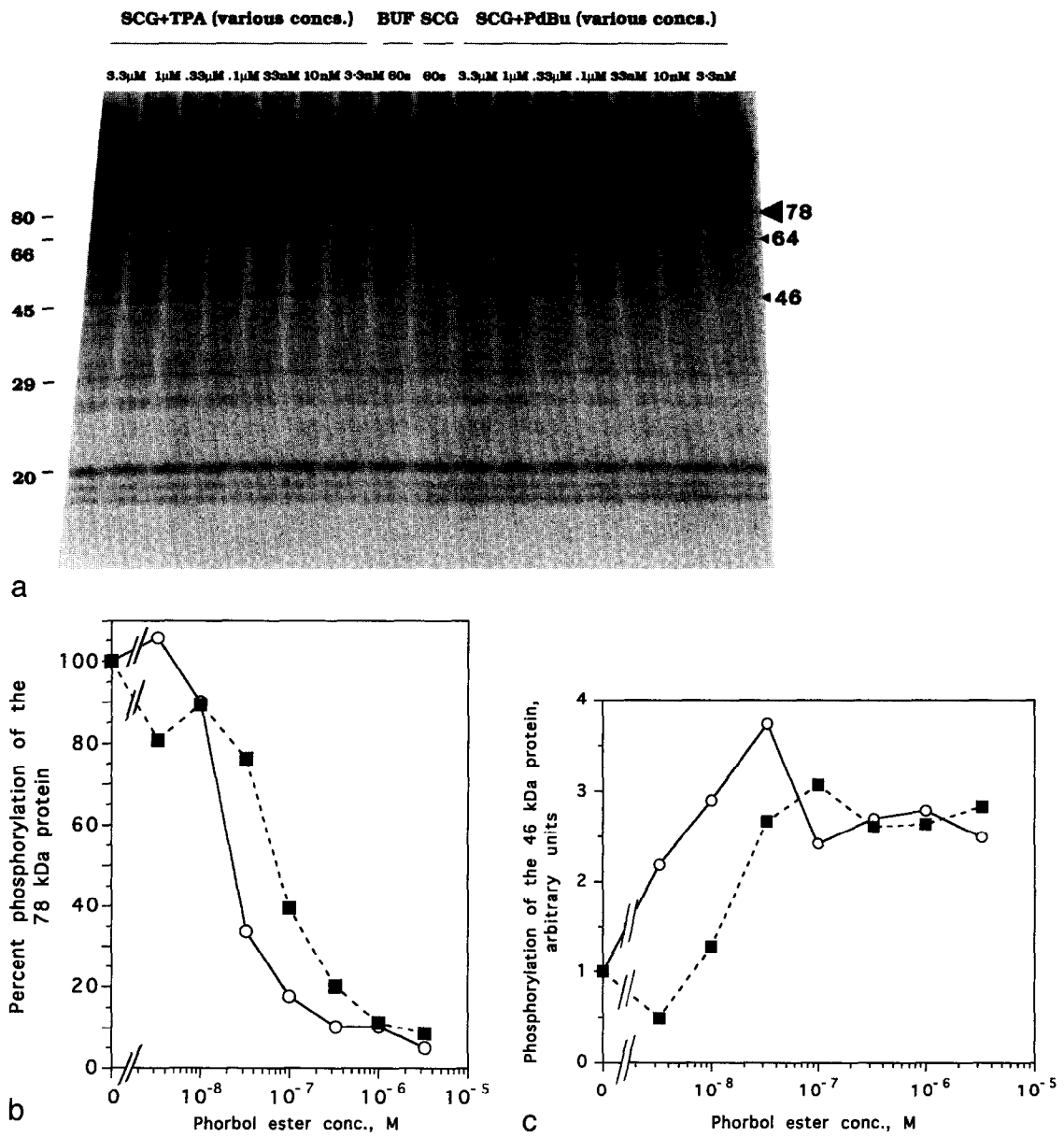

FIG. 5. Protein phosphorylation changes in rat mast cells on exposure to different concentrations of TPA and PdBu in the presence of cromolyn. (a) Autoradiograph. Rat mast cells were labelled with [^{32}P]orthophosphate and exposed for 60 sec to TPA and PdBu over the concentration range of 3.3 nM to 3.3 μM and including the simultaneous addition of 100 μM cromolyn (final concentration). Samples were analysed by SDS-PAGE and the resultant autoradiograph is shown. Figures on the left indicate molecular mass markers in kDa. Arrowheads indicate the positions of the 78-, 64- and 46-kDa phosphoproteins. (b) Phosphorylation of the 78-kDa protein as assessed by densitometer scanning of the autoradiograph shown in (a). The extent of phosphorylation induced by 100 μM cromolyn alone is given a value of 100%. cromolyn + TPA (○), cromolyn + PdBu (■). (c) Phosphorylation of the 46-kDa protein as assessed by densitometer scanning of the autoradiograph shown in (a). The extent of phosphorylation in the presence of buffer alone is given a value of 1.0. cromolyn + TPA (○), cromolyn + PdBu (■).

to the activation of a phosphatase, which act on the 78-kDa protein. Other possible explanations for the dephosphorylating effect of phorbol esters include direct inhibition of the 78-kDa protein kinase, activation of the 78-kDa protein phosphatase, or indeed, different cellular targets [40–43]. The use of phosphatase inhibitors may help to clarify any involvement of a phosphatase in mediating the effect of phorbol esters on cromolyn-induced phosphorylation. Interestingly, two previous reports have suggested a role for a protein phosphatase in the regulation of secretion since okadaic acid, an inhibitor of type-1 and -2A phosphatase, was found to inhibit IgE-mediated histamine release in rat peritoneal mast cells [44, 45].

Currently, at least 10 different isoforms of PKC are now known and have been classified into three groups named as classical, non-classical and atypical, depending on their various sensitivities to, or requirements for calcium, phospholipid and phorbol esters [46]. Classical (α, β_I, β_{II} and γ)

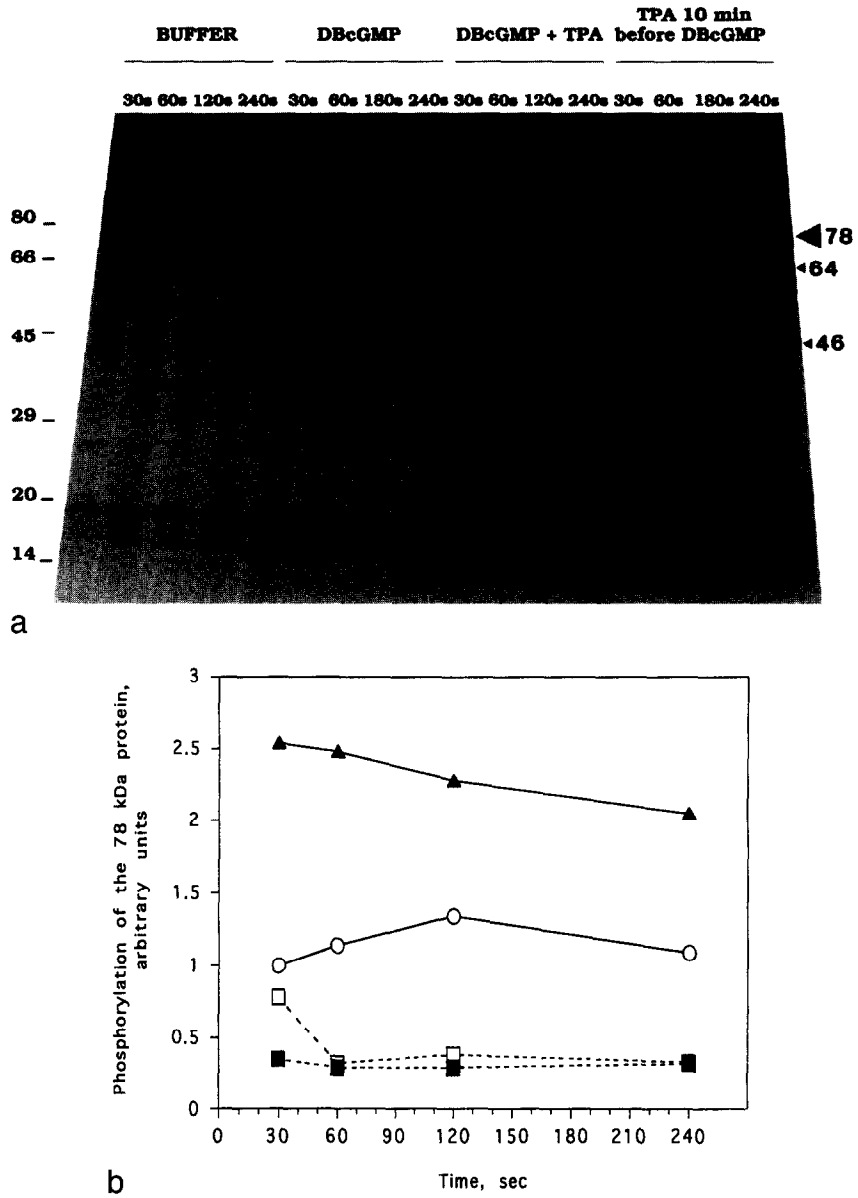

FIG. 6. Inhibition of dibutyryl cyclic GMP-induced phosphorylation of the 78-kDa protein by TPA. (a) Autoradiograph. Rat mast cells were labelled with [^{32}P]orthophosphate and the phosphorylation assay performed as described. Samples were removed for analysis at 30, 60, 120 and 240 sec after the addition of buffer alone, DBcGMP (1 mM, final concentration), DBcGMP (1 mM, final concentration) with TPA (150 nM, final concentration). A fourth treatment involved a 10 min pre-incubation with 200 nM TPA followed by addition of DBcGMP (1 mM, final concentration) and aliquots removed for analysis as above. The resultant autoradiograph is shown with figures on the left indicating molecular mass markers in kDa and arrowheads indicating the positions of the 78-, 64- and 46-kDa phosphoproteins. (b) Phosphorylation of the 78-kDa protein as assessed by densitometer scanning of the autoradiograph shown in (a). The extent of phosphorylation in the presence of buffer alone at 30 sec is given a value of 1.0. THG buffer (○), DBcGMP (▲), DBcGMP added with TPA (□), TPA added 10 min prior to DBcGMP (■).

and non-classical (δ, ϵ, η and θ) PKC isoforms are activated by phorbol esters whereas atypical isoforms (ζ, λ and ι) are unaffected. However, to date, no PKC isoforms have been shown to be inhibited by phorbol esters. These findings, taken together with our observation that PKC-activating phorbol esters do not stimulate phosphorylation of the 78-kDa protein, suggest that a PKC isoform of the classical or non-classical type is unlikely to be the kinase which phosphorylates the 78-kDa protein, but do not exclude involvement of atypical PKC subtypes. In the secretory, rat basophilic RBL-2H3 cell line it is clear that several different PKC isoforms may be activated during secretion and play different roles. The presence of PKC isoforms α, β, δ, ϵ and ζ has been demonstrated in RBL-2H3 cells and all of these, except ζ, shown to be activated upon cell stimulation by antigen or TPA [15]. Furthermore, PKC isozymes β and δ may be involved in stimulating antigen-induced secretion [15], whereas isozymes α and ϵ may be involved in feedback inhibition of phospholipase C [47]. Thus, different PKC isozymes may have specific roles in regulating various

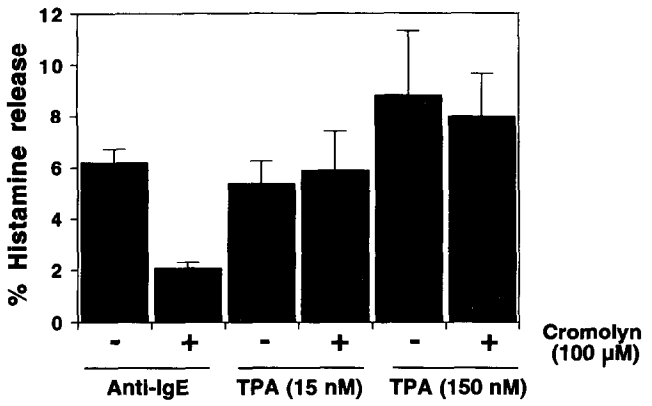

FIG. 7. Effect of cromolyn on histamine release induced by anti-IgE and TPA. Rat peritoneal mast cells were stimulated with anti-IgE (1.7 μg/mL) or TPA (15 or 150 nM) in the presence or absence of cromolyn (100 μM) and the histamine release determined as described. Results shown are the means (± SEM) of triplicate determinations from three separate experiments.

processes involved in secretion in RBL-2H3 cells, but whether this is the case in rat peritoneal mast cells is less clear.

We also report here that TPA also blocked DBcGMP-induced phosphorylation of the 78-kDa protein suggesting that TPA must act at a point subsequent to a presumed rise in intracellular cGMP [18]. However, cGMP itself (rather than the cell permeant derivative, DBcGMP) has recently been shown to inhibit mast cell secretion and postulated to act through the same site as cromolyn, on the mast cell surface [48]. We also have data which suggest that cromolyn does not elevate mast cell cGMP levels (manuscript in preparation) and thus the effect of TPA on 78-kDa protein phosphorylation, whether stimulated by cromolyn or DBcGMP, is likely to be through the same mechanism.

Recently, the isolation and partial amino acid sequencing of the phosphorylated 78-kDa protein demonstrated an homology to moesin, a cytoskeletal component belonging to the band 4.1 superfamily of proteins [37]. Currently, moesin has no known function, but Correia and co-workers suggest that, in its phosphorylated state, the 78-kDa protein may help to form a rigid cytoskeletal network which prevents secretion from taking place. Dephosphorylation of the 78-kDa protein was speculated to bring about disassembly of cytoskeletal components, by an unknown mechanism, allowing secretion to proceed. Clearly, many cell types express moesin but, to date, only mast cells have been shown to phosphorylate this protein in response to cromolyn. The mechanisms involved in regulating the phosphorylation of the 78-kDa protein, how these are perturbed by cromolyn (and phorbol esters), and the target protein to which cromolyn binds are important issues that have yet to be resolved.

In summary, we have demonstrated a novel and unexpected action of phorbol esters in the potent inhibition of cromolyn-induced phosphorylation of a 78-kDa mast cell protein. This indicates that phorbol ester-sensitive PKC is not the kinase responsible for phosphorylating the 78-kDa protein, although involvement of an atypical PKC cannot be excluded. However, a phorbol ester-sensitive PKC may regulate dephosphorylation of the 78-kDa protein by an as yet unknown mechanism. The failure of cromolyn to inhibit phorbol ester-induced histamine release further supports a link between cromolyn inhibition of secretion and phosphorylation of the 78-kDa protein.

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