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Characterization of the 78 kDa Mast Cell Protein Phosphorylated by the Antiallergic Drug Cromolyn and Homology to Moesin

*Ivan Correia, Linghua Wang, Xinzhu Pang and Theoharis C. Theoharides**
DEPARTMENT OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, TUFTS UNIVERSITY SCHOOL OF MEDICINE, BOSTON, MA 02111, U.S.A.

ABSTRACT. Mast cells (MC) can be stimulated to secrete by cross-linking immunoglobulin E bound to specific surface receptors, as well as in response to polycationic molecules such as substance P and compound 48/80. The antiallergic drug disodium cromoglycate (cromolyn) inhibited MC secretion and rapidly incorporated phosphate into a 78 kDa protein, speculated to be its mode of action. This protein was purified by single and two-dimensional gel electrophoresis, and was shown to be phosphorylated primarily on serine residues by protein kinase C. Partial amino acid sequencing of two generated fragments was identical to that of portions of mouse moesin, a member of the band 4.1 superfamily of proteins, with no definitive function known to date. Polyclonal antibodies raised against the rat basophil leukemia cell moesin cDNA expressed in *Escherichia coli* immunoprecipitated the 78 kDa phosphoprotein quantitatively, and immunocytochemistry localized it to the plasma membrane. Reversible phosphorylation of this 78 kDa phosphoprotein could affect its possible cytoskeletal binding through which it may regulate stimulus–secretion coupling in MC. BIOCHEM PHARMACOL 52;3:413–424, 1996.

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

MC† express on their surface the IgE binding protein (FcεRI), which has high affinity for the Fc portion of IgE [1]. Receptor-bound IgE, when bridged by multivalent antigen, triggers an integrated non-cytolytic series of biochemical reactions that involve activation of protein tyrosine kinases [2]. These include the *src*-related $p56^{lyn}$ and $p72^{syk}$ of which the first phosphorylates the β and γ subunits of FcεRi and the syk tyrosine kinase itself. In addition, syk then possibly leads to the phosphorylation of phospholipase C γ1 and 2 (PLC), which are then translocated from the cytosolic to the membranous fraction and lead to 1,4,5-inositol triphosphate and diacylglycerol production. These events, in turn, lead to an increase in free intracellular calcium and activation of PKC, which somehow leads to further phosphorylation of FcεRI on threonine of the γ chains and serine on the β-subunits [1, 2]. The net result is the release of many mediators stored in their cytoplasmic granules by degranulation or compound exocytosis. These include histamine, heparin, proteases, and cytokines, as well as the *de novo* synthesis and release of mediators such as prostaglandins, leukotrienes, lipoxins, and platelet-activating factor [3, 4]. MC are also activated by diverse stimuli such as lymphokines and cytokines, naturally occurring or synthetic basic peptides, lectins, hormones, some neuropeptides and neurotransmitters, as well as direct nerve stimulation [5]. Therefore, MC are considered important not only for allergic and late phase reactions, but also for many inflammatory conditions such as migraines, interstitial cystitis, and irritable bowel syndrome [4–6].

Much is known about the role of signaling molecules such as G proteins [7] and calcium [8] in exocytosis. There now exists strong evidence that MC exocytosis involves G proteins [9] and that polycationic compounds interact directly with G proteins [10]. Specifically, the monomeric G_{i3} protein [11] and the peptide Rab3a [12] appear to be involved in exocytosis. However, molecules involved in its termination remain unknown, even though the phosphorylation state of a putative molecule was proposed using MC as a model for secretion [13]. MC loaded with $^{32}P_i$ and stimulated either with C48/80 [14], with a neuropeptide [15], or immunologically [16] incorporated phosphate into three proteins of molecular mass 68, 59 and 42 kDa within 15 sec, which corresponded to the time–course of secretion. The 59 kDa protein has now been identified as vimentin, an intermediate filament protein [17]. A fourth 78 kDa protein was phosphorylated at about 2 min and was speculated to be the physiological mechanism for termination of secretion [14].

* Corresponding author: T. C. Theoharides, Ph.D., M.D., Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Tel. (617) 636-6866; FAX (617) 636-6456.

† *Abbreviations:* BME, β-mercaptoethanol; C48/80, compound 48/80; cromolyn, disodium cromoglycate; IEF, isoelectric focusing; IgE, immunoglobulin E; MARCKS, myristoylated alanine-rich C kinase substrate; MC, mast cell(s); PK, protein kinase; PVDF, polyvinylidene difluoride; and RBL, rat basophilic leukemia.

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This same 78 kDa protein is phosphorylated when MC are treated with cromolyn [18], a "membrane MC stabilizer," which does not cross the plasma membrane [19], but can inhibit both antigen and C48/80-induced MC secretion [20]. The concentration range at which cromolyn facilitated the incorporation of phosphate into the 78 kDa protein corresponded to that which inhibited histamine release; moreover, both phosphorylation and inhibition of histamine release showed tachyphylaxis [18]. These findings were reproduced independently [16], and were extended to cromolyn's analogue nedocromil [21] and to structurally related compounds such as quercetin [22]. We, therefore, undertook to characterize the 78 kDa protein phosphorylated by cromolyn.

MATERIALS AND METHODS

Reagents

All reagents were obtained from the Sigma Chemical Co. (St. Louis, MO), unless stated otherwise. Cromolyn, a bischromone [1,3-bis-(2-carboxychromon-5-yloxy)-2-hydropropane], also was purchased from Sigma.

Purification of Peritoneal MC

MC were obtained by lavage of the peritoneum of male 325 g rats (Taconic Farms, Germantown, NY) injected with Locke's buffer (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5 mM HEPES, 1 g/L dextrose and 1 g/L BSA, pH 7.2). Cells were then purified to greater than 90% purity by centrifugation at 350 *g* for 10 min at room temperature through metrizamide (Accurate Chemical & Scientific Corp., Westbury, NY) [18].

Culture of RBL Cells

RBL cells were obtained from Dr. H. Metzger (NIH) and were maintained as described before [23].

Protein Assay

The Pierce BCA Protein Assay kit was used for the spectrophotometric determination (at 562 nm) of protein concentration. For small samples (5–250 μg range), the enhanced protocol using 60° for 30 min was employed.

Radiolabeling of Cells with $^{32}P_i$

Cells were labeled at 10 μCi/million cells/mL with ^{32}P-carrier free orthophosphoric acid (ICN-Biomedicals, Costa Mesa, CA) in phosphate-free Locke's buffer for 1 hr at 37°, were washed twice with Locke's without BSA, and were resuspended in the same buffer. They were treated with 10^{-4} M cromolyn for 30 sec at 37°.

One-Dimensional Electrophoresis (SDS–PAGE)

(A) The discontinuous buffer system developed by Laemmli [24].

(B) The method of Schägger and von Jagow [25] for separation of peptides of low molecular mass (5–20 kDa) [25].

Two-Dimensional Electrophoresis for IEF

(A) O'Farrell protocol [26].

SAMPLE PREPARATION WITH PHENOL EXTRACTION. Cells were first lysed in 0.5 mL of extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2% BME and 2 mM phenylmethylsulfonyl fluoride) and were sonicated with multiple short bursts of maximum intensity [27]. The volume was adjusted to 1.5 mL and incubated for 10 min on ice; then an equal volume of water-saturated phenol was added. After 10 min with shaking at room temperature, the phases were separated by centrifugation. Proteins were precipitated from the phenol phase with 5 vol. of 0.1 M ammonium acetate in methanol and kept at −20° overnight. The precipitate was washed three times with ammonium acetate and once with acetone. The pellet was then dried under nitrogen gas and was solubilized in O'Farrell's buffer [0.5% SDS, 9.5 M urea, 2% ampholytes (pH 3.5–10) from Bio-Rad Laboratories (Hercules, CA) and 5% BME]. After 10 min, an equal volume of Garrel's buffer [9.5 M urea, 2% ampholytes (pH 3.5–10), 4% NP-40, and 5% BME] was added.

COMPOSITION OF FIRST-DIMENSIONAL GELS. IEF gels were composed of 4% bis-acrylamide, 8.0 M urea, 4% NP-40, 2% ampholytes (pH 3.5–10), 0.14% *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and 0.14% ammonium persulfate. Protein samples (about 100 μg) were loaded per cylinder gel at the basic end and allowed to electrophorese at 400 V for 9000 VHr. The voltage was then increased to 800 V for 1 hr. Next, the gels were extruded and equilibrated in 10 mL of equilibration buffer for 15 min and were either placed directly onto a second-dimensional gel or frozen at −20°. Two additional tube gels were also run along with the samples. They were extruded and cut at 1-cm intervals. Each piece was equilibrated in distilled water, and the pH of these solutions was then measured and plotted relative to the length of the tube gel to determine the pIs of the proteins.

RESOLUTION IN THE SECOND DIMENSION. Resolution was on 7.5% SDS–PAGE gels overlaid with 1% agarose stacking gel containing 0.125 M Tris (pH 6.8). The second-dimensional gels were run overnight at 50 V (constant voltage) until the tracking dye ran off the bottom. The gels were dried immediately and placed against film. The molecular masses of the protein were determined by prestained molecular weight markers (Sigma).

(B) Imada and Sueoka discontinuous gel system [28].

This system was designed for the separation of poorly soluble, hydrophobic cell surface proteins using SDS and Triton CF10 detergents, and urea in the first dimension. In the second dimension, proteins were separated by their molecular masses.

COMPOSITION OF THE FIRST-DIMENSIONAL GEL. The detergents 0.1% SDS and 0.3% Triton CF10 (alkyaryl ether) were present in both the stacking gel (1.8% acrylamide, 0.18% bis-acrylamide, Tris 0.125 M, pH 6.8) and the separating gel (3% acrylamide, 0.3% bis-acrylamide, urea 9 M, Tris 0.375 M, pH 9). The anode buffer contained 375 mM Tris (pH 9), and the cathode buffer 50 mM glycine-NaOH (pH 10.5).

SAMPLE PREPARATION. Cells were lysed and solubilized in SDS solubilizing buffer (2% SDS, 0.0625 M Tris, 10% glycerol, 5% BME), and the samples were boiled for 3–5 min. Then, they were applied directly to the gel and overlaid with the cathode buffer, which was cooled before use. Further separation was carried out at a constant voltage of 200–300 V for 3–4 hr. The gels were extruded and analyzed immediately in the second dimension as described for the IEF method.

Electroelution

The autoradiograph was matched with the gel, and the desired molecule to be electroeluted was marked out. The excised gel was suspended briefly in water to separate gel from backing paper and to rehydrate the gel. Tris–glycine–SDS buffer was used to elute proteins into Centricon-30 tubes (Amicon, Beverly, MA). Voltage of 150–250 V was applied for 20 hr. Then the sample was washed twice using 1.5 mL of volatile buffer (50 mM ammonium bicarbonate and 0.1% SDS).

Chemical Cleavage by Cyanogen Bromide

The sample was washed twice with volatile buffer (5 mM NH_4HCO_3, 0.1% SDS) in a final volume of 75 μL. An amount of 40 μL of 0.15 M CNBr in 70% formic acid was added and incubated overnight in the dark at room temperature. The sample was dried on a Speed-Vac and solubilized in sample buffer (50 μL).

Western Blotting

Transfer of proteins from gel to nitrocellulose membranes was by semi-dry blotting. The transfer buffer contained 25 mM Tris, 190 mM glycine and 20% methanol. Transfer was typically carried out at 20 V for 25 min. The blocking of non-specific binding sites on the membrane was carried out using 3% BSA in PBS. Primary rabbit anti-rat moesin antibody was typically used at a dilution of 1:2000 with overnight shaking. Secondary antibody used was anti-rabbit IgG horseradish peroxidase (Zymed, San Francisco, CA). Detection was carried out with diaminobenzidine.

Electroblotting

The method of Matsudaira [29] was used where proteins were electroblotted directly onto PVDF. The gel was first soaked in transfer buffer [10 mM 3-(cyclohexylamino)-1-propane sulfonic acid, 10% methanol, pH 11] for 5 min to reduce the amount of Tris and glycine. PVDF membrane was then rinsed in 100% methanol and stored in transfer buffer. The proteins were electroblotted onto PVDF. Then the PVDF membrane was washed in deionized water for 5 min, stained with 0.1% Coomassie Blue R-250 in 50% methanol for 5 min, and subsequently destained in 50% methanol, 10% acetic acid for 5–10 min. The membrane was rinsed in water, air-dried at room temperature, and autoradiographed; the spot of interest was cut out for sequencing.

Peptide Sequencing

Peptide sequencing was carried out by automated cycles of the Edman degradation reaction using the Applied Biosystems model 477 Pulsed Liquid Sequencer at the Tufts University Protein Sequence Facility.

Phosphoamino Acid Analysis

The 78 kDa phosphoprotein was digested exhaustively with 50 μg/mL trypsin, 50 μg/mL chymotrypsin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in 25 mM ammonium bicarbonate for 18 hr at 37°. Then the supernatant was lyophilized, resuspended in about 20 μL of water, and digested in a Kimble tube with 6.0 N HCl at 100° for 2–3 hr. The digested sample was resuspended in the pH 1.9 buffer tank (2% formic acid, 8% acetic acid) and spotted on TLC plates along with phenol red as a tracer. Also spotted was about 1 μL of a 10 mg/mL stock solution of phosphothreonine/serine/tyrosine along with phenol red. The TLC plate was then wetted with the pH 1.9 buffer. The first "half" dimension was run at 500 V until tracer dye was 2 cm short of the apex of the bent TLC plate. At this point, the plate was transferred to the pH 3.5 tank (10% acetic acid, 1% pyridine) and run at 400 V until tracer dye was about 3 cm from the edge of the plate. The plate was then removed and left to dry. Ninhydrin (0.25%) in n-butanol was used to develop the standards. The plates were then autoradiographed, and the sample was compared to the standards.

Kinase Inhibitors

Radiolabeled MC were pretreated with different concentrations and for different times with various cell permeable serine/threonine kinase inhibitors which included PKA, PKG, PKC and calcium/calmodulin kinase II (CAMK II) (BIOMOL Research Laboratories, Plymouth Meeting, PA), followed by cromolyn for 30 sec at 37°, lysed, and run on one-dimensional SDS–PAGE. The relative specificity (K_i in μM) of these inhibitors was as follows: chelerythrine (PKC, 0.66), H-8 (PKA, 1.2; PKG, 0.48; PKC, 15), H-89

(PKA, 0.048; PKG, 0.48; PKC, 31.7; CAMK II 29.7), KN-62 (CAMK II, 0.9), and staurosporine (PKA, 0.007; PKG, 0.0085; PKC, 0.0007).

Antibodies

The RBL moesin gene was subcloned into pET-28 vector and transformed into *Escherichia coli* JM109 (DE3); after isopropyl-β-D-thiogalactopyranoside (IPTG) induction, a fusion protein with polyhistidine on the N-terminus was expressed.* The protein was purified to homogeneity using a nickel column under denaturing conditions. The purified protein was injected into rabbits, and polyclonal antisera were generated by Immuno Dynamics (San Diego, CA) and used without further purification [30]. Rabbit polyclonal anti-calf moesin serum was supplied by Dr. Furthmayr. It cross-reacted with both ezrin and moesin [31] and recognized two bands in RBL cell lysate. A monoclonal antibody that recognizes both ezrin and moesin was purchased from Zymed.

Immunoprecipitation

MC were loaded with $^{32}P_i$, treated with cromolyn, and boiled for 5 min in 1% SDS. Double-strength inhibitor solution (PBS containing 20 mM NaPP, 100 mM NaF, 2 mM EGTA, 2 mM EDTA, 5% NP40, and protease inhibitors at a final concentration of 10 μg/mL) was added to the sample at 1:1 dilution. Proteins in the lysate were precleared by incubation for 20 min with 50 μL of Protein A linked to Sepharose. The clear supernatant was transferred to 10 μL of the rabbit anti-rat moesin polyclonal antibody and incubated for 30 more min. To the reaction mixture, 50 μL of Protein A linked to Sepharose was added and incubated for another 30 min and spun at 10,000 *g* for 1 min. The pellet was washed twice with 1 mL of SII (150 mM NaCl, 15 mM HEPES, 1 mM EDTA, 0.5% NP-40, pH 7.4). To the pellet, 100 μL of solubilizing SDS buffer was added, boiled for 5 min, and loaded onto SDS–PAGE gels [30].

Immunocytochemistry

Purified MC were treated with 100 μM cromolyn for 30 sec and were fixed immediately in 4% paraformaldehyde. Frozen sections were cut at 7 μm and treated with a 1:200 dilution of rabbit anti-rat moesin polyclonal antibody at room temperature for 1 hr. Then the sections were incubated with a 1:200 dilution of goat anti-rabbit IgG-biotin (Vector Laboratories, Burlingame, CA) for 30 min, followed by a further exposure to streptavidine-rhodamine (Pierce, Rockford, IL) for 30 min. Next the sections were mounted in aqueous mounting medium and observed under a light microscope (Nikon, Don Santo Corp., Natick, MA).

* Wang L, Correia I, Pang X, Wang Y and Theoharides TC, Manuscript submitted for publication.

RESULTS

Purification and N-Terminal Sequencing of the 78 kDa Phosphoprotein

In radiolabeled MC treated with cromolyn (100 μM), a 78 kDa protein was rapidly phosphorylated within 30 sec and was completely dephosphorylated in 5 min (Fig. 1). Two-dimensional IEF was first used to purify this protein, but the 78 kDa phosphoprotein demonstrated considerable charge heterogeneity (results not shown). Solubilization with NP-40 and 3-[3-cholamido-propyl)dimethylammonia]-1-propane sulfonate (CHAPS) was not adequate, while loading the protein sample at the acidic end and reversing the polarity of the electrophoretic run (NEPHGE gels) also failed to introduce the protein fully into the gel. Extraction in phenol prevented aggregation and permitted solubiliza-

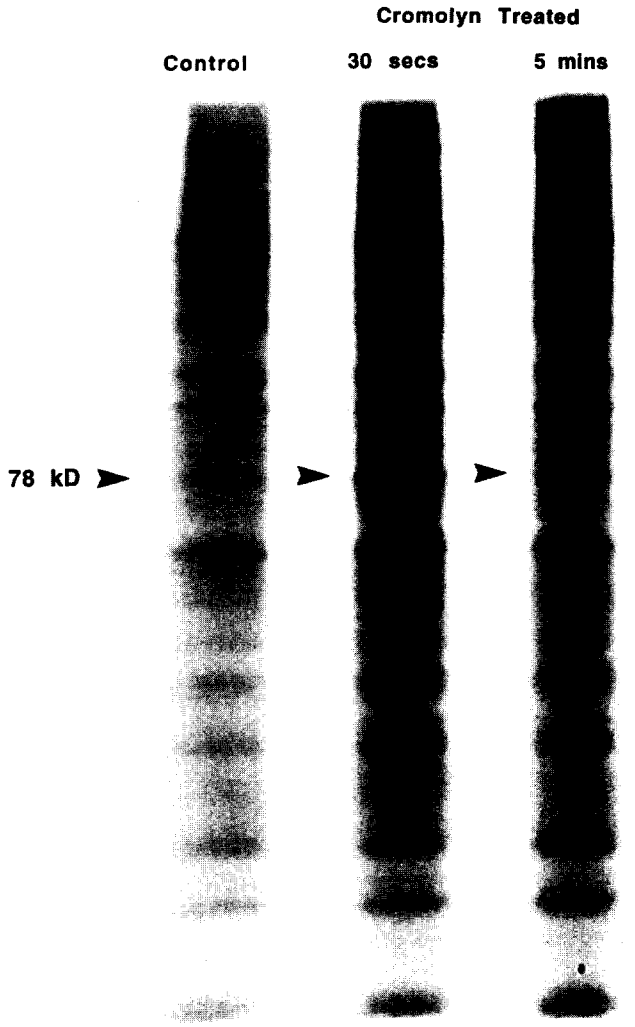

FIG. 1. Endogenous protein phosphorylation of the 78 kDa protein. Control and cromolyn-treated MC were labeled with $^{32}P_i$ as described in Materials and Methods. Cells were then incubated with cromolyn (100 μM) for 30 sec and 5 min, immediately lysed, and run on one-dimensional SDS–PAGE. Exposure of film was for 24 hr at −70°. Arrows indicate the position of 78 kDa protein.

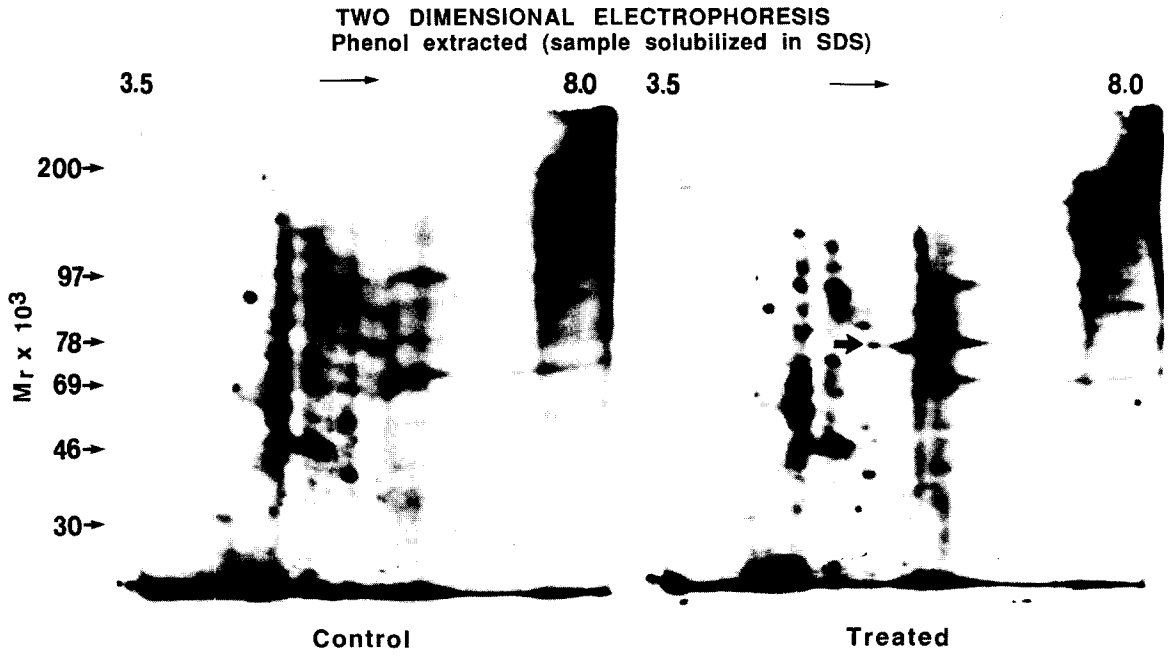

FIG. 2. Two-dimensional IEF analysis. Protein samples extracted using phenol were subsequently solubilized in SDS and NP-40 (1:8) and run on two-dimensional IEF gels as described in Materials and Methods. Exposure of film was for 24 hr at -70°. Molecular weights of standard protein markers in kDa are indicated on the left side and the established pH gradient at the top of the figure. The arrow inside the gel indicates the position of the 78 kDa phosphoprotein.

tion in SDS (Fig. 2), but due to the numerous steps involved, the 78 kDa phosphoprotein was finally solubilized directly in SDS and purified on an alternate two-dimensional gel system (Fig. 3). This protocol separated proteins primarily on the basis of their hydrophobicity in the first dimension and molecular mass in the second dimension. Sufficient quantity (40–60 pmol) of protein was obtained for sequencing.

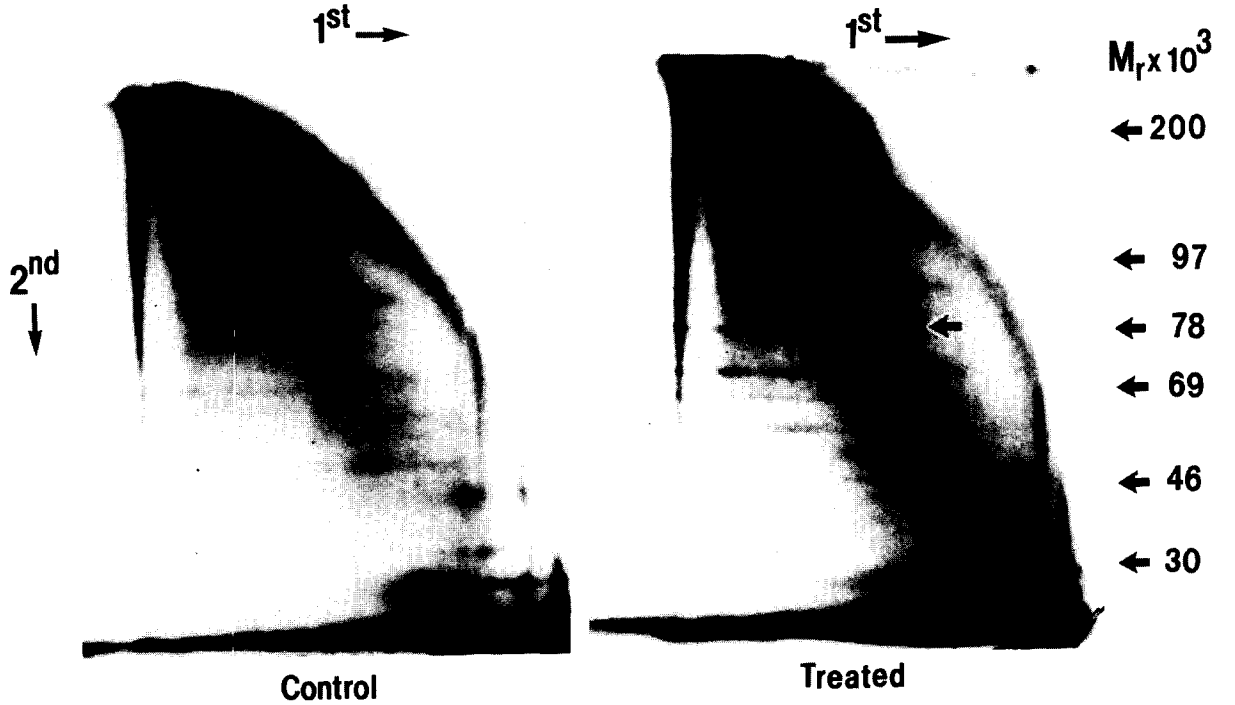

FIG. 3. Two-dimensional PAGE using detergents in the first dimension. Control and cromolyn-treated ("treated") MC were solubilized in SDS and directly analyzed as described in Materials and Methods. Exposure of film was for 24 hr at -70°. The arrows at the right-hand side indicate the position and size (in kDa) of the molecular weight markers used. The dark arrow against the white background inside the gel identifies the position of the 78 kDa phosphoprotein.

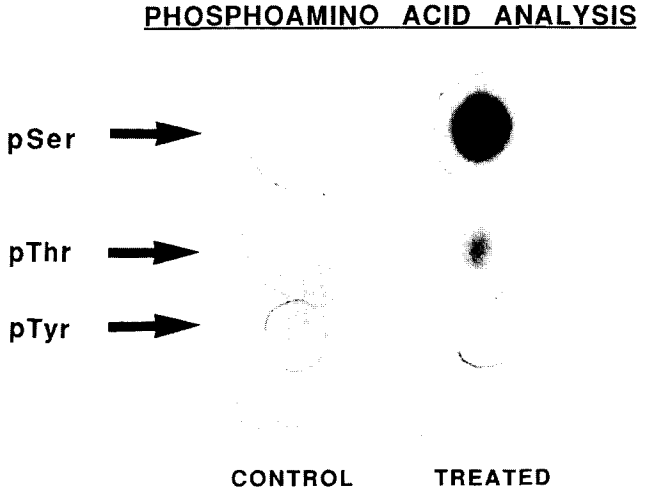

FIG. 4. Phosphoamino acid analysis of the 78 kDa protein. The phosphorylated protein was exhaustively digested, and phosphoamino acids were separated by thin-layer chromatography as described in Materials and Methods. Exposure of film was for 72 hr at -70°. Circles indicate the positions of standards stained with ninhydrin. Arrows indicate the amino acids recognized by this method.

Partial Amino Acid Sequence

Internal sequence was obtained by electroelution of the phosphoprotein and cyanogen bromide treatment. The fragments generated were separated on a 10% tricine–SDS–PAGE system, electroblotted onto PVDF, stained, and autoradiographed. Two *radiolabeled* fragments of molecular masses 18 and 24 kDa were cut out and sequenced. Twelve amino acids from the N terminus of the 24 kDa fragment (M, D, A, E, L, E, F, A, I, Q, P, and N) showed 100% homology to the N terminus 12–23 of mouse moesin, ezrin, and radixin. Sixteen amino acids from the N terminus of the 18 kDa fragment (M, E, R, A, L, L, E, N, E, K, K, K, R, E, L, and A) showed 100% homology to region 318–333 of mouse moesin, 81% to mouse radixin, and 64% to mouse ezrin.

Phosphoamino Acid Analysis

Phosphoamino acid analysis revealed that after cromolyn treatment moesin was phosphorylated primarily on serine (with much less on threonine) residues (Fig. 4), thus implicating a serine/threonine kinase and phosphatase in the regulation of its phosphorylation.

Inhibitors of Serine/Threonine Protein Kinases

Pretreatment with chelerythrine which is selective for PKC prevented incorporation of phosphate into the 78 kDa protein by cromolyn. Pretreatment at 100 μM for 1 hr with

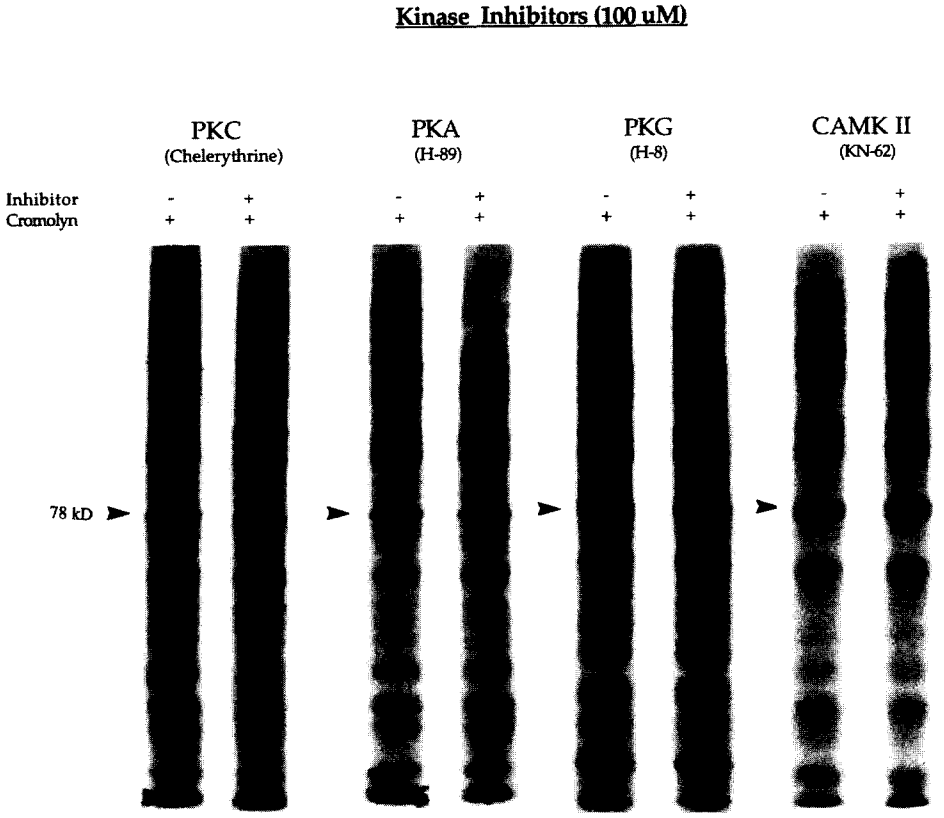

FIG. 5. Effect of kinase inhibitors on the phosphorylation of the 78 kDa protein. Radiolabeled MC were pretreated with a 100 μM concentration of the protein kinase inhibitors H-89, H-8, KN-62 and chelerythrine for 1 hr, with or without incubation with cromolyn for an additional 30 sec at 37°. The MC were then lysed and run on SDS–PAGE as described in Materials and Methods. The relative specificity (K_i in μM) of these inhibitors for the various types of protein kinases is listed in Materials and Methods. The arrowhead indicates the position of the 78 kDa phosphoprotein. Exposure of film was for 24 hr at -70°.

H-8 which is selective for PKA and PKG, H-89 which is selective for PKA, and KN-62 which is selective for CAMK II had no effect on 78 kDa phosphorylation (Fig. 5). Pretreatment with sphingosine (100 μM for 2 min), a specific inhibitor of PKC, and staurosporine (0.1 μM for 15 min), a frequently used potent but non-specific PKC inhibitor, also prevented completely the incorporation of phosphate into the 78 kDa protein (see Fig. 6). These results implicated PKC in cromolyn's phosphorylation of the 78 kDa protein.

Specificity of Anti-Rat Moesin Polyclonal Antibody

Western analysis performed on total RBL lysate with anti-rat moesin serum recognized a single band (see Fig. 7, lane 1). Immunoprecipitation of RBL cell lysate with anti-rat moesin and probing with a polyclonal anti-calf moesin/ezrin serum (supplied by Dr. Furthmayr) demonstrated that the anti-rat moesin serum immunoprecipitated only moesin (see Fig. 7, lane 2). In contrast, the anti-calf moesin/ezrin serum recognized two bands in RBL cell lysate (Fig. 7, lane

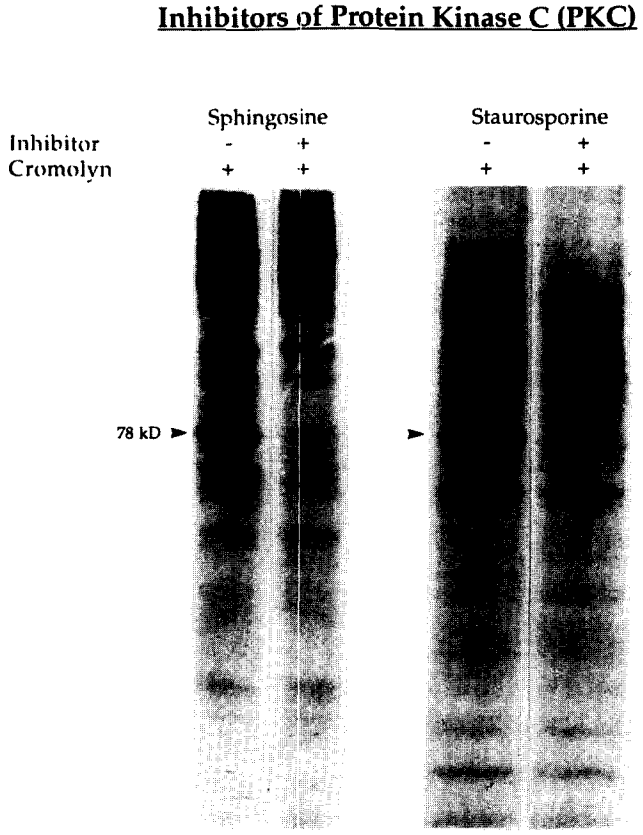

FIG. 6. Effect of PKC inhibitors on the phosphorylation of the 78 kDa protein. Radiolabeled MC were pretreated with sphingosine (100 μM for 2 min) or staurosporine (0.1 μM for 15 min), with or without incubation with cromolyn for an additional 30 sec at 37°. The MC were then lysed and run on one-dimensional SDS–PAGE as described in Materials and Methods. The arrowhead indicates the position of the 78 kDa phosphoprotein. Exposure of film was for 24 hr at 70°.

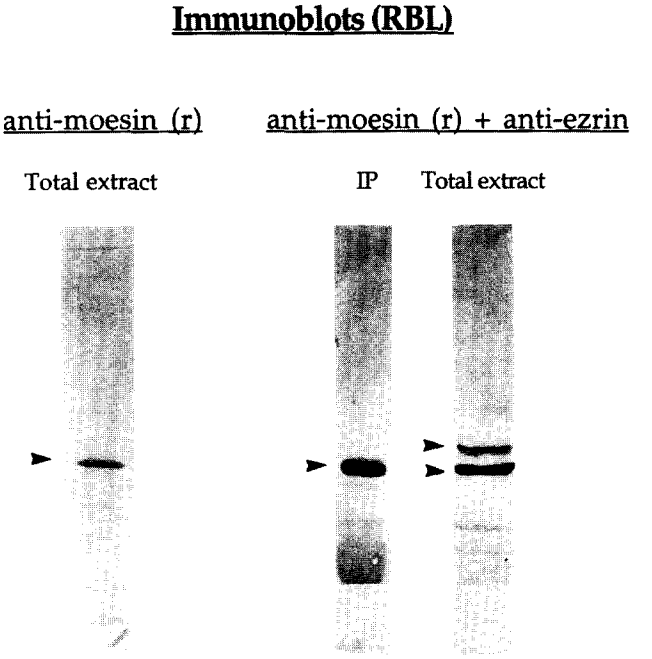

FIG. 7. Specificity of the anti-rat moesin serum. Immunoblots were performed on RBL cells as described in Materials and Methods. Lane 1: Anti-rat moesin serum recognized a single band on total RBL lysate; no cross-reactivity with ezrin was observed. Lane 2: Anti-rat moesin serum immunoprecipitated only moesin from RBL cell lysate, subsequently probed with anti-moesin and anti-ezrin serum. Lane 3: RBL cell lysate probed with both anti-rat moesin and anti-ezrin serum show the presence of both ezrin (upper arrowhead) and moesin (lower arrowhead).

3). Western blotting of total MC lysate with a monoclonal serum that recognized both moesin and ezrin showed that the prominent band in RBL cells was ezrin, while in MC it was moesin (Fig. 8).

Immunoprecipitation

Radiolabeled control or cromolyn-treated MC extracts were first immunoprecipitated with anti-rat moesin serum and the precipitate was analyzed by SDS–PAGE. Autoradiography revealed a single phosphorylated 78 kDa protein (Fig. 9). Immunoprecipitation removed all of the 78 kDa phosphoprotein from the supernatant (Fig. 9), thus indicating that the phosphoprotein could not be simply "co-precipitated" along with moesin.

Localization

The specific anti-rat moesin clearly localized the 78 kDa protein at discrete punctate structures at the plasma membrane, and no cytoplasmic staining was observed (Fig. 10A). Treatment with cromolyn (Fig. 10B) did not alter its distribution.

DISCUSSION

Cromolyn is used for the prophylactic treatment of allergic asthma, rhinitis, and conjunctivitis, conditions associated

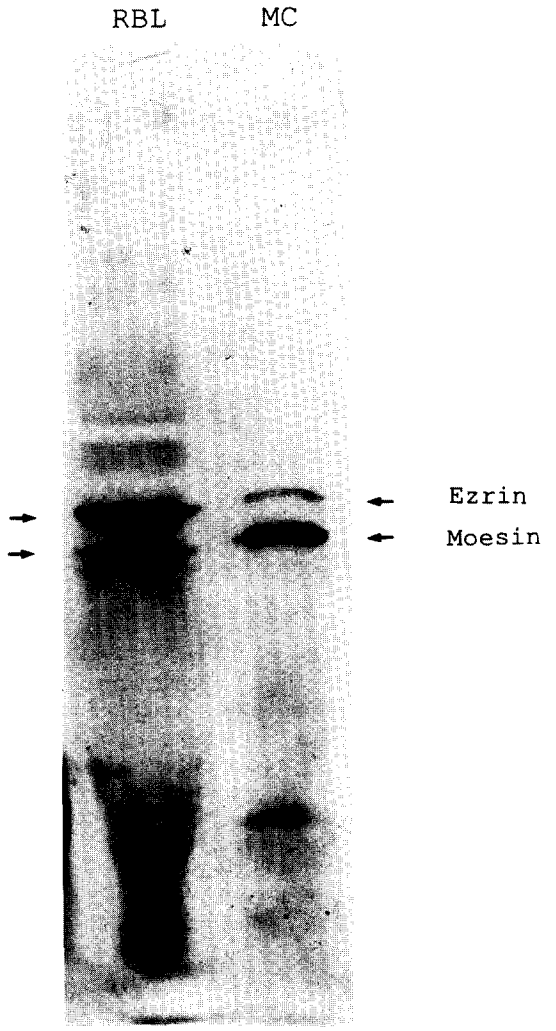

FIG. 8. Investigation of the presence of ezrin and moesin in MC and RBL cells. Immunoblots were performed on MC and RBL cells as described in Materials and Methods using a monoclonal antibody that recognizes both ezrin and moesin. Lane 1: RBL cells. Lane 2: MC. The arrows indicate the position of ezrin and moesin.

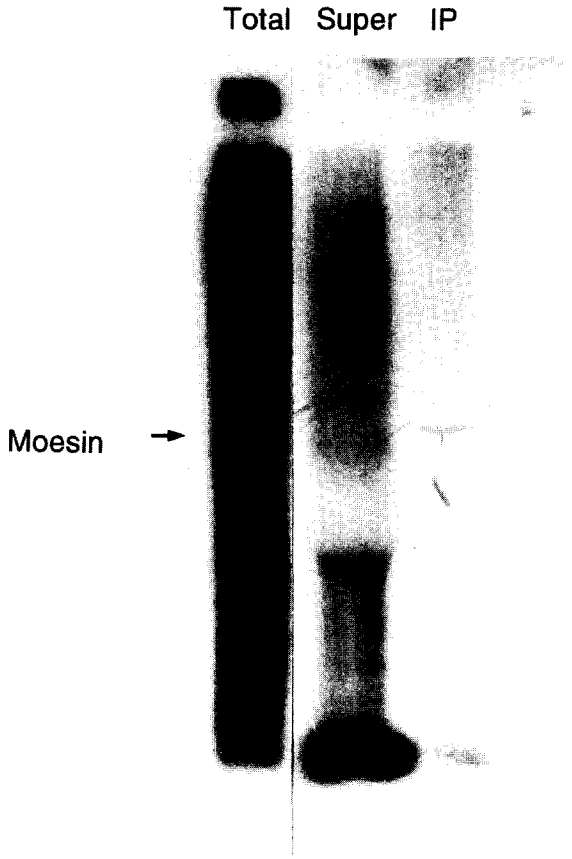

FIG. 9. Immunoprecipitation of the 78 kDa phosphoprotein. Extracts from cromolyn-treated $^{32}P_i$-labeled MC were precipitated with anti-rat moesin serum and separated into supernatant and precipitate fractions as described in Materials and Methods. Exposure of film was for 72 hr at −70°. The arrow indicates the position of moesin. Super = supernatant; IP = immunoprecipitate.

with MC degranulation [32]. Despite years of research, the precise mechanism of action for cromolyn is not understood completely [33]. It is thought to somehow stabilize the MC plasma membrane and prevent the release of mediators both after immunologic challenge [34, 35] or after stimulation with C48/80 [36, 37]. Cromolyn exerts its action from the cell surface, as it cannot cross the plasma membrane and enter the cell [19]. Earlier studies focused on its ability to modulate calcium influx via a putative calcium channel [38–40]. However, cromolyn can inhibit MC secretion stimulated via C48/80 in the absence of extracellular calcium [36, 37] and by suboptimal doses of the calcium ionophore A23187 [20, 36], which speak against the cromolyn receptor–calcium channel hypothesis. In other studies, a cell permeable form of cromolyn inhibited RBL secretion through inhibition of a nucleoside 5′-diphosphate kinase, suggesting that a surface receptor may not be required [41]. In light of the controversial results mentioned above, the ability of cromolyn to increase phosphate incorporation into the 78 kDa protein [18] remains the most attractive hypothesis on its mode of action [33].

The 78 kDa phosphoprotein is now shown to have phosphopeptide sequences that are identical to the N-terminal sequences 12–23 and 318–333 of mouse moesin [31]. Identification of a single band of 78 kDa after quantitative immunoprecipitation with rabbit anti-rat moesin serum strongly suggests that the 78 kDa protein acquiring phosphate in response to cromolyn is moesin or a protein with high homology to it. Moesin was first purified from bovine uterus on a heparin-Sepharose column, and was thought to act as a receptor for heparin [42]. The human gene encoding this protein was cloned [31], and its sequence demonstrated no signal peptide or transmembrane domains, suggesting that the protein was not integrally incorporated into the membrane. Moesin belongs to a family of proteins also referred to as the band 4.1 superfamily [43]. Members of this superfamily include ezrin (73% identity), radixin (80% identity), and merlin (50% identity). They all share a highly homologous N-terminal domain, which is postulated to interact with the cytoplasmic domain of the plasma

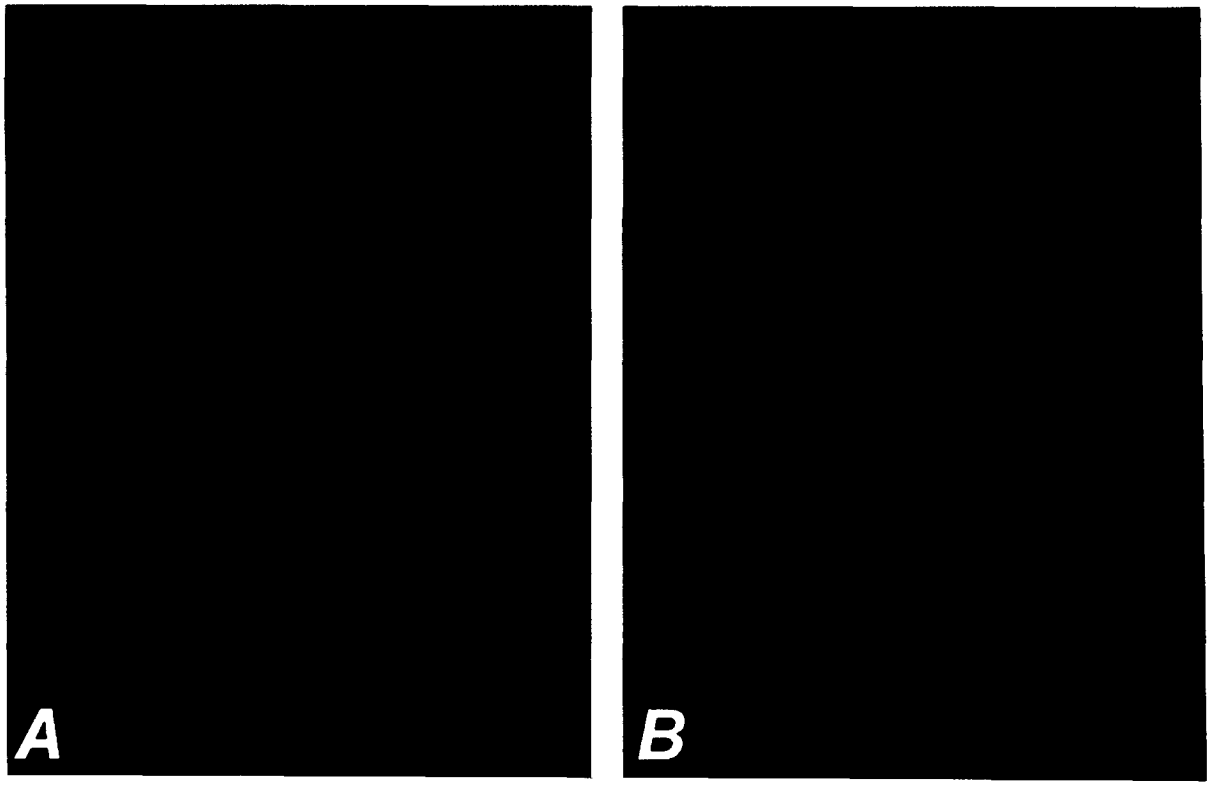

FIG. 10. Immunocytochemistry of moesin. Purified MC were prepared as described in Materials and Methods. The primary antibody was used at a dilution of 1:200, and detection was with streptavidin-rhodamine as described in Materials and Methods. (A) control, untreated MC. (B) MC treated with cromolyn (10 μM for 30 sec).

membrane, while the carboxyl termini are quite variable and may determine the function of these proteins. Immunocytochemistry demonstrated the localization of the 78 kDa protein at the cytoplasmic site of the plasma membrane of peritoneal MC.

Here we show that cromolyn induced phosphorylation of the 78 kDa protein primarily on serine residues by PKC, and the two labeled peptides sequenced from two non-overlapping regions indicate at least *two* sites phosphorylated *in vivo*. These may be on serines 56 or 74 and/or threonine 66 and serine 374 of the radiolabeled fragments sequenced. Moesin is widely distributed in many cell types [43], but there has been no report on its possible phosphorylation to date. The functions of many other members of the band 4.1 superfamily however, are, regulated via phosphorylation. For instance, band 4.1 phosphorylation by multiple kinases, including cyclic AMP kinase, PKC and membrane casein kinase, regulates its affinity for the cytoskeleton [44–46]. Band 4.1 is related to synapsin I found in nerve terminals where it has been shown to regulate secretion [47]. Ezrin, which has been localized in actin-containing surface structures, such as microvilli and membrane ruffles [48], is phosphorylated by epidermal growth factor both on Tyr-145 and Tyr-353 in human A-431 cells [48, 49]. However, in parietal cells, ezrin is phosphorylated on serine residues during secretion [50], suggesting that phosphorylation on different residues of the same molecule may be associated with different functions of the protein. In fact, the tissue distribution of ezrin and moesin is distinctly different, with ezrin present in most cells, while moesin is found primarily on endothelial cells and leukocytes [51, 52].

Radixin was originally isolated as an F-actin capping protein in hepatic adherens junction and focal contacts of cultured cells [53]. During mitosis, this protein is localized to the cleavage furrow [53, 54]. Merlin, another member of this family, also known as Schwannomin, has been demonstrated to have suppressor activity for neurofibromatosis-2 [55, 56], and it is interesting to note that MC have been associated with neurofibroma growth [57].

Sagi-Einsenberg [58] had speculated that in MC one PKC form could be involved in the inhibition, while another in the activation of secretion. She further speculated that cromolyn specifically *inhibited* the PKC isoform involved in the activation of secretion, thus permitting the inhibitory pathway to proceed through the phosphorylation of the 78 kDa protein, identified earlier [18]. Our data appear to suggest, instead, that cromolyn *activates* a specific isoform of PKC, of which as many as ten different isoforms have been identified to date [59] and have a central role in transducing both stimulatory and inhibitory signals. In fact, RBL cells contain the calcium-dependent isoforms (α and β), the calcium-independent isoforms (δ and ε), and the atypical isoform (ζ [60, 61]. The β and δ isoforms are involved in the activation of secretion [60, 61], whereas the α and ε isoforms are involved in the inhibition of secretion [60, 61]. This dual action of PKC provides a versatile regu-

latory system that may be finely tuned by second messengers. One part of this control may involve phosphorylation of the FcεRI, possibly through β and δ isoforms [1, 2]. However, phosphorylation of moesin also occurs in response to C48/80, indicating that it does not depend on FcεRI-related phosphorylation events.

The exact mode of action of moesin in regulating MC secretion is not known. Disassembly of the cortical F-actin network is recognized as an important event prior to secretion in many cell types [62–64]. Synapsin I interacts with F-actin *in vitro*, but phosphorylation by at least three different kinases dissociates it from actin, which frees secretory vesicles to undergo exocytosis [65]. In its dephosphorylated state, myristoylated alanine-rich C kinase substrate (MARCKS) protein is bound to the plasma membrane and cross-links actin; phosphorylation by PKC upon activation permits the movement of MARCKS to the cytosol, thus permitting neurosecretion [66].

Members of the ERM family have all been shown to be colocalized with actin. A highly conserved 25 amino acid region in the C-terminus region of ezrin, radixin, and moesin reportedly acts as an actin-binding domain in ezrin [67]. Radixin is also known to bind actin in the C-terminus [53], and it is speculated that this actin binding domain is masked and that unmasking is regulated by phosphorylation [67]. Radixin has been shown to bind directly to the barbed ends of actin filaments [54, 68] whereas ezrin is only capable of binding actin indirectly via an unidentified protein [69] which may involve heterotypic associations with moesin [70, 71]. Such association may also occur between moesin molecules [70] and appears to involve binding of an N-terminal domain to a C-terminal domain that is normally masked [72, 73]. In fact, moesin and ezrin were shown to be associated with actin in cultured cells [48] and in neutrophil plasma membranes [52]. In its phosphorylated state, this 78 kDa phosphoprotein could self-associate in a multivalent complex [70], establishing a rigid network of cytoskeletal proteins cross-linked with the plasma membrane. Thus. the actin network could act as a physical barrier to granule exocytosis. Dephosphorylation of the 78 kDa protein could somehow lead to disassembly of the cortical F-actin network, thus permitting exocytosis.

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