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PROTEIN PHOSPHORYLATION IN RAT MAST CELL GRANULES

CYCLIC AMP DEPENDENT PHOSPHORYLATION OF A 44K PROTEIN ASSOCIATED WITH BROKEN GRANULES

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Abstract—When rat mast cells are prelabeled with $^{32}PO_4$ and exposed to non-immunologic or immunologic stimuli under conditions that lead to mediator release from granules, they show rapid increases in labeling of a number of high molecular weight proteins. To determine if granule membrane proteins are subject to protein phosphorylation and perhaps participate in this response, granules with intact or broken membranes were isolated from sonicated, purified rat serosal mast cells on a Percoll gradient. When the granules with broken membranes were incubated with [γ-^{32}P]ATP and Mg^{2+} in the absence of exogenous protein kinases, one major radioactive band was recovered in the 44K area after electrophoresis in sodium dodecyl sulfate/polyacrylamide gels. The phosphorylation reaction with ATP required Mg^{2+}, was enhanced by 0.05 to 0.5 μM cyclic AMP, and was inhibited by Ca^{2+} (0.5 mM and higher). The initial reaction was rapid, and the maximal response was seen at 30°. The 44K band was absent in granules with intact membranes incubated with [γ-^{32}P]ATP but present when intact granules were lysed with distilled water before adding the [γ-^{32}P]ATP. These observations indicate that granules have an endogenous phosphorylating system and that the phosphorylation response is on the inner surface of the granule membranes. The possibility was not excluded that a portion of the phosphorylating activity was derived from the cytosol and became firmly associated with broken granules when the intact cells were sonicated. Analysis for possible phosphorylated amino acids in the 44K band after acid hydrolysis showed both phosphorserine and phosphothreonine, indicating that the radioactivity was in a phosphorylated protein or glycoprotein. The 44K phosphorylated protein was made up of several components ranging in pI from approximately 7.6 to 6.6. While the identity of the phosphorylated 44K polypeptide is uncertain, one important possibility is that it is part of an autophosphorylated cAMP dependent protein kinase. The cyclic AMP dependent phosphorylating activity present in granules provides a mechanism by which the granules might respond rapidly to cyclic AMP during mediator release.

The secretion of granule mediators from mast cells is a critical step in hypersensitivity responses involving IgE antibodies in the skin and lung. Granule exocytosis occurs by the fusion of cytoplasmic granule membranes with each other and with plasma membrane, permitting the release of granules or their contents into the medium. Recent studies in our laboratory have indicated that phosphatidylinositol kinase, an enzyme which catalyzes the formation of diphosphoinositide from phosphatidylinositol, is a component of rat mast cell granules. Through its effects on granule membrane charge this enzyme may play an important role in the rapid changes in granule structure and distribution associated with secretion [1]. Since membrane protein phosphorylation regulates many physiologic processes [2] including membrane permeability and transport [3, 4], the possible involvement of protein phosphorylation in the granule response must also be seriously considered.

Several reports have suggested that changes in protein phosphorylation may be involved in immunologic and non-immunologic activation of mediator release from rat mast cells [5–8]. Studies in intact mast cells labeled with $^{32}PO_4$ and activated by the divalent cation ionophore A23187 indicate that a number of proteins [5, 6] are rapidly phosphorylated. One of these is the 45–62K glycoprotein component of the receptor for IgE immunoglobulin [6] which is also rapidly phosphorylated when mast cells are stimulated immunologically by cross-linked IgE molecules bound to the cell surface [7]. Studies on broken mast cells have shown that these cells contain both cyclic AMP dependent [8] anbd calcium-activated, phospholipid-dependent protein kinases [9]. While these observations suggest a role for changes in protein phosphorylation in secreting mast cells, nothing is known about whether or not the phosphorylation response involves proteins in the mast cell granule itself.

In the present report, granules were studied for the possible presence of endogenous phosphorylating activity. Evidence will be presented that at least

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one protein in mast cell granules with an apparent molecular weight of 44K in sodium dodecyl sulfate/polyacrylamide gels was phosphorylated in the presence of ATP and Mg^{2+} provided the granules were broken or lysed. The phosphorylation occurred in the absence of exogenous enzymes or protein substrates. These observations provide evidence for an endogenous phosphorylating system within the granule.

MATERIALS AND METHODS

Reagents and their sources were as follows: [γ-^{32}P]ATP (>4000 Ci/mmol; ICN Pharmaceuticals, Irvine, CA); [^{32}P]-orthophosphoric acid (carrier free) in HCl-free water (New England Nuclear, Boston, MA); bovine serum albumin (BSA*), adenosine 3′:5′-cyclic monophosphate (cyclic AMP), guanosine 3′:5′-cyclic monophosphate (cyclic GMP), trizma base, Percoll, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2-mercaptoethanol (2-ME), Nonidet P-40, ethylene glycol-bis-(β-aminoethyl ether) *N,N*′tetra acetic acid (EGTA), phosphatidylserine, diolein, phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), dimethyl sulfoxide (DMSO), Brilliant Blue G, standard phosphoprotein and ninhydrin (Sigma Chemical Co., St. Louis, MO); trichloroacetic acid (TCA), calcium chloride, sodium chloride, magnesium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, ammonium bicarbonate, acetone, ethanol, methanol, ether, toluene, sodium (tetra) ethylenediamine tetra acetate (EDTA), pyridine, acetic acid glacial and glycerol (Fisher Scientific Co., Fair Lawn, NJ); heparin sodium (O'Neal, Jones & Feldman, St. Louis, MO); cellulose MN 300 (0.1 mm) thin-layer chromatography (TLC) plate (Brinkmann, Westbury, NY); sodium dodecyl sulfate (SDS) (Gallard-Schlesinger, Carle Place, NY); acrylamide, *N,N*′-methylene-bisacrylamide (bisacrylamide), ammonium persulfate, *N,N,N*′,*N*′-tetramethyl-ethylenediamine, bromophenol blue and Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA); ampholines (LKB Instruments Inc., Rockville, MD); and urea (Schwarz/Mann Inc., Spring Valley, NY).

Rat mast cell preparations and isolation of rat mast cell granules

Rat serosal cells were obtained by peritoneal and pleural lavage of male Sprague–Dawley rats (250–300 g; Camm, Wayne, NJ) with heparinized (10 units/ml) balanced salt solution (BSS) (4.0 mM Na_2HPO_4, 2.7 mM KH_2PO_4, 150 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, pH 7.2) with 0.046% (w/v) BSA (BSSA). Mast cells were purified on a Percoll gradient as described [9]. The mast cells were more than 90% pure by toluidine blue and other dye staining reactions.

Rat mast cell granules were separated in a Percoll gradient by the method of Lagunoff and Pritzl [10] with minor modifications. The characterization of the different granule fractions has been described in detail by these authors. Mast cell granules obtained by this method are distributed in two distinct bands in the gradient: one band close to the top (band A, broken granules) and one band close to the bottom of the gradient (band C, intact granules). The two bands, A and C, and the intermediate column (band B) were collected separately. Electron microscopy and ruthenium red staining revealed that band A consisted largely (more than 90%) of swollen granules lacking membranes and band C largely (more than 95%) of granules with intact membranes.

Incubation procedures

Unless otherwise indicated, granules were washed twice with BSS after separation. Phosphorylation was routinely carried out at 30°. A 50-μl sample of resuspended granules in BSS was diluted to a final volume of 100 μl in a reaction mixture containing 1 mM $MgCl_2$, 20 mM Tris/HCl (pH 7.5) with or without other reagents such as cyclic nucleotides and protease inhibitors (unless otherwise indicated each tube contained granules from 1.5 to 2.6 × 10^6 mast cells, uncorrected for losses during purification). Phosphorylation was started by the addition of 10 μl of 100 μM [γ^{32}P]ATP (diluted with unlabeled ATP to 5 × 10^9 cpm/μmole) and allowed to proceed for 5 min. Control incubations contained 2 mM EDTA instead of Mg^{2+}. The reaction was terminated by the addition of TCA to a final concentration of 10% (w/v) with rapid mixing by vibration. After 30 min at 4°, the samples were centrifuged at 750 *g* for 20 min at 4° and resuspended in 0.1 ml of chilled distilled water; 1.9 ml of chilled acetone was added and the mixture was incubated for 30 min at 4° and then centrifuged at 12,000 *g* for 20 min. The supernatant fraction was discarded, and the pellet was boiled for 5 min in 70 μl of 3 × disintegration buffer consisting of 2% (w/v) SDS, 10% (w/v) glycerol, 0.001% (w/v) bromophenol blue and 0.25 M Tris/HCl (pH 6.8) with or without 5% (w/v) 2-ME.

^{32}P-*Incorporation into mast cell granules lysed in distilled water*

Intact membrane granules (from 2.0 × 10^6 mast cells, purity 90.5%) washed twice with BSS after separation were suspended in 10 ml of chilled distilled water and agitated briefly by vibration. The suspension was centrifuged at 60 *g* for 8 min at 4° and the pellet was resuspended in BSS. The granules were treated with either one or two cycles of this procedure. After this step, mast cell granule phosphorylation was carried out under the standard conditions in the presence or absence of 5 mM EGTA.

* Abbreviations: BSA, vobine serum albumin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PIPES, piperazine-*N,N*′-bis (2-ethanesulfonic acid); EGTA, ethylene glycol-bis-(β-aminoethyl ether) *N,N*′-tetra acetic acid; EDTA, sodium (tetra) ethylenediamine tetra acetate; 2-ME, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate; cyclic AMP, adenosine 3′:5′-cyclic monophosphate; cyclic GMP, guanosine 3′:5′-cyclic monophosphate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate;DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; bisacrylamide, *N,N*′-methylene-bisacrylamide; BSSA, balanced salt solution with BSA; BSS, balanced salt solution without BSA; and MCM, mast cell medium.

Gel electrophoresis

One-dimensional. SDS/polyacrylamide-slab-gel electrophoresis was performed by the method of Laemmli [11]. A linear gradient of polyacrylamide, from 6 to 18% (w/v), was used as the separating gel with a 3% (w/v) acrylamide with 2.7% (w/w) bisacrylamide for the stacking gel. In all gels the ratio of acrylamide to bisacrylamide was 37:1 by weight. Gels were fixed and stained in a solution containing 12% (w/v) TCA, 50% (v/v) methanol and 0.03% (w/v) Coomassie Brilliant Blue R-250. Gels were destained with several changes of 7% (w/v) acetic acid/10% (v/v) methanol. The stained and washed gels were soaked in 7% (v/v) acetic acid/1% (w/v) glycerol/10% (v/v) methanol and dried on filter paper under vacuum. To determine the incorporation of ^{32}P, autoradiographs were prepared by exposing the dried gels to Kodak XR-5 medical X-ray film. The film was processed with Kodak RPX-Omat developer and scanned in a Zeineh Soft Laser scanning densitometer (Biomed Instruments Inc., Chicago, IL).

For estimation of the molecular weights of phosphorproteins in SDS/polyacrylamide gels, the following standards were employed (molecular weights in parentheses): chicken skeletal-muscle myosin (200,000), *Escherichia coli* β-galactosidase (116,250), rabbit skeletal-muscle phosphorylase B_4 (92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400).

Two-dimensional. Two-dimensional SDS/polyacrylamide-slab-gel electrophoresis was performed by the method of O'Farrell [12]. Isoelectric focusing was carried out in rods (105 mm × 5 mm) containing 9.2 M urea, 2% (w/v) Nonidet P-40, 2% ampholines (composed of 1.6% pH range 5 to 8 and 0.4% pH range 3.5 to 10). The gels were polymerized with 3.8% (w/v) acrylamide and 5.8% (w/w) bisacrylamide. After isoelectric focusing, some of the gel tracks were cut into 5-mm slices and eluted with double-distilled water for pH measurements. The second dimension was the discontinuous SDS/polyacrylamide-slab-gel electrophoresis system of Laemmli [11]. The separating gel consisted of a linear gradient of polyacrylamide from 18% (w/v) to 6% (w/v) acrylamide with 2.7% (w/v) bisacrylamide and the stacking gel consisted of 3% (w/v) acrylamide with 2.7% (w/v) bisacrylamide. Gels were fixed, stained, destained, and dried, and autoradiographs were prepared as described for one-dimensional gels.

Analysis of phosphorylated amino acids

Protein was extrated from gels in 0.05 M NH_4HCO_3 containing 0.1% SDS and analyzed for phosphorylated amino acids as described by Beeman and Hunter [13]. The protein was precipitated by 20% TCA at 4°, washed successively with ethanol and ethanol/ether (1:1), suspended in 6 N HCl and hydrolyzed at 100° for 3 hr in tubes sealed under bacuum. The HCl was removed by evaporation. The hydrolysates wee dissolved in a marker mixture containing unlabeled phosphoserine, phosphothreonine and phosphotyrosine (1 mg/ml each) and analyzed on TLC plates by electrophoresis at pH 3.5 (acetic acid/pyridine/H_2=, 1:10:189) for 90 min at 1.2 kV. The markers were detected by staining with ninhydrin.

Protein assay of granules

Protein concentrations of granules were determined by the method of Bradford [14] using crystalline BSA as a standard.

Standardization of granule concentrations

Concentrations of broken and intact granules were standardized with respect to one another on the basis of major granule membrane proteins common to both types of granules. After SDS/polyacrylamide-slab-gel electrophoresis of granule proteins, stained proteins of each lane were scanned in a Zeineh Soft Laser scanning densitometer.

Protein kinase assays

Protein kinase C and cAMP-dependent protein kinase activities were assayed on intact granules by measuring the incorporation of ^{32}P from [$\gamma^{32}P$]ATP into lysine-rich histone. The conditions below were found to be optimal for quantitation based on prelimary studies with cytosol preparations from mast cells or RBL-1 cells under various conditions of time, temperature and enzyme concentrations. In measurements of protein kinase C activity, the reaction mixture contained 0.5 mM $CaCl_2$, 5 mM $MgCl_2$, 20 mM Tris/HCl (pH 7.5), 6 μg/ml 1,2-diacylglycerol (as the diolein derivative), 24 μg/ml phosphatidylserine, 200 μg/ml lysine-rich histone, 10 μM [^{32}P]ATP (diluted to 5×10^7 cpm/μmole with unlabeled ATP) and intact granules from 2×10^6 cells. Lipids were emulsified in 20 mM Tris/HCl (pH 7.5) by sonification immediately before addition to the phosphorylation mixture. After incubation for 3 min at 30°, the reaction was stopped by the addition of 100 μg BSA and 2 ml of 25% (w/v) TCA. Precipitates were washed twice with 3 ml of 25% TCA, dissolved in 500 μl of NCS tissue solubilizer and radioactivity was measured by liquid scintillation counting.

Cyclic AMP dependent protein kinase activity was measured in granules under the same conditions except that the reaction mixture contained 10 mM $MgCl_2$ instead of Ca^{2+}, no lipids and 5 μM cyclic AMP.

RESULTS

^{32}P Incorporation into granule proteins

As shown previously by Lagunoff and Pritzl [10], unreduced and reduced proteins in intact and broken granules from unstimulated mast cells can be resolved by electrophoresis in SDS/polyacrylamide gels into several discreet bands in the 26–40K range which stain prominently with Coomassie Brilliant Blue, as well as some additional less prominently stained areas (Fig. 1). Since broken granules lose large amounts of soluble proteins from the inside of the granules when lysis occurs, these major granule membrane proteins were useful in comparing intact and broken membrane granule preparations with one another with respect to their relative membrane concentrations.

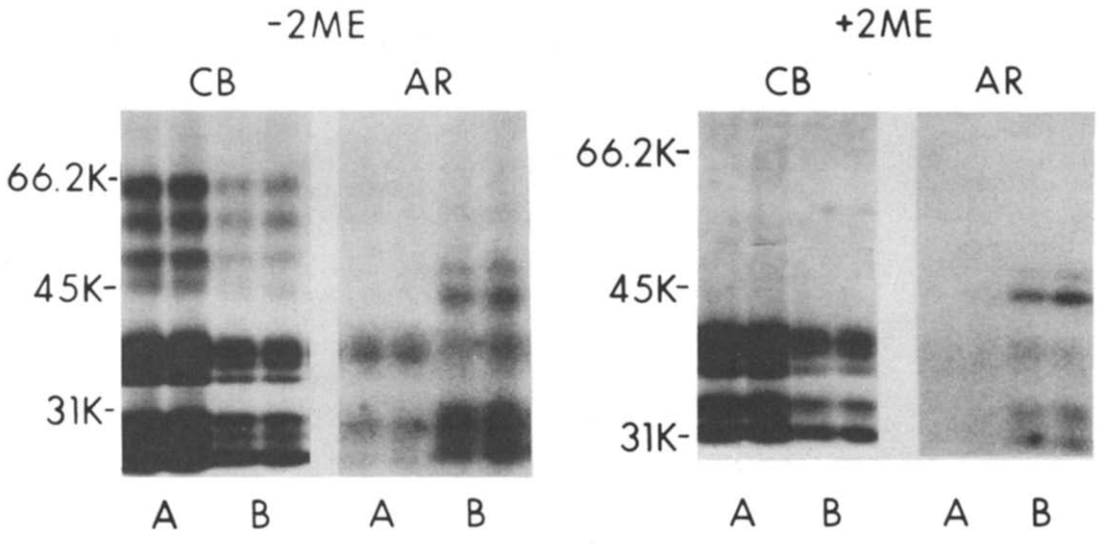

Fig. 1. One-dimensional SDS/polyacrylamide-gel electrophoresis of rat mast cell granules with intact membranes and broken membranes. (A) Granules with intact membranes washed twice with BSS after isolation from the Percoll gradient (2.6×10^6 mast cell equivalents); (B) granules with broken membranes washed twice with BSS after isolation from the Percoll gradient (2.6×10^6 mast cell equivalents), and containing approximately 20% of the membrane protein content of A as determined by scanning of stained proteins of electrophoresed samples. Samples were incubated with [γ-^{32}P]ATP as described in Materials and Methods. Abbreviations: CB, Coomassie Brilliant Blue stain, and AR, autoradiograph. Numbers at left indicate the molecular weights of standards listed in Materials and Methods. Duplicate lanes are shown.

Intact membrane granules incubated with [γ-^{32}P]ATP and Mg^{2+} developed two broad and two narrow radioactive bands corresponding to the major granule proteins. However, almost all of the radioactivity was removed by precipitation with TCA and extraction with acetone. Moreover, when each of the proteins in these bands was hydrolyzed with 6 N HCl for 3 hr at 100° and analyzed by electrophoresis on TLC plates, no phosphoserine, phosphothreonine or phosphotyrosine was detected. Thus, it appeared that the ^{32}P was not incorporated covalently into these proteins.

The results with broken granules labeled under the same condition and at approximately 20% of membrane protein concentrations differed in that, in addition to the reversible major granule protein labeling, a discreet, relatively prominent labeled band was present at the 44K area in both reduced and unreduced gels (Figures 1 and 2). The radioactivity in this area was not removed by TCA and acetone. In Coomassie Brilliant Blue stains of the electropherogram, a very weakly staining band was observed in the 44K area irrespective of the presence of 2-ME. The phosphorylation in this area was not detected in intact membrane granule preparations, even in overexposed films. Under the same labeling conditions a second weaker radioactive band not seen with granules with intact membranes was often identified in the 46K area (Fig. 2).

Acid hydrolysis and electrophoresis of ^{32}P-labeled proteins

After hydrolysis of the 44K protein in broken granules with 6 N HCl and electrophoretic separation on TLC plates [13], 42% of the ^{32}P was recovered in phosphoserine and phosphothreonine (Fig. 3). Since yields of phosphoamino acids from phosphorylated proteins are usually in the range of 30–45% following acid hydrolysis, these observations indicate that the phosphorylation is occurring on aliphatic hydroxyl groups of a protein. By contrast, attempts to demonstrate phosphorylated amino acids in hydrolyzed samples from the 46K band have been unsuccessful, and the nature of the phosphate binding is currently unknown.

The following experiments to characterize the 44K protein phosphorylation were carried out in reduced gels.

^{32}P-Incorporation into intact membrane granules lysed in distilled water

Since the inner as well as the outer granule membrane is exposed in broken granules, the possibility was considered that cytoplasmic proteins might bind more effectively to broken than to intact granules and provide either phosphorylating enzyme, the 44K protein substrate that is phosphorylated, or both. If this were true, granules that had been isolated as intact granules, carefully washed and then deliberately broken would not be expected to show the phosphorylation response. To evaluate this question, washed granules with intact membranes were lysed in distilled water and incubated with [γ-^{32}P]ATP in the presence of $MgCl_2$. Granules with intact membranes with two cycles of this hypotonic treatment produced definite although less marked ^{32}P incorporation into the 44K band in the presence of 5 mM EGTA, as is true in granules with broken membranes

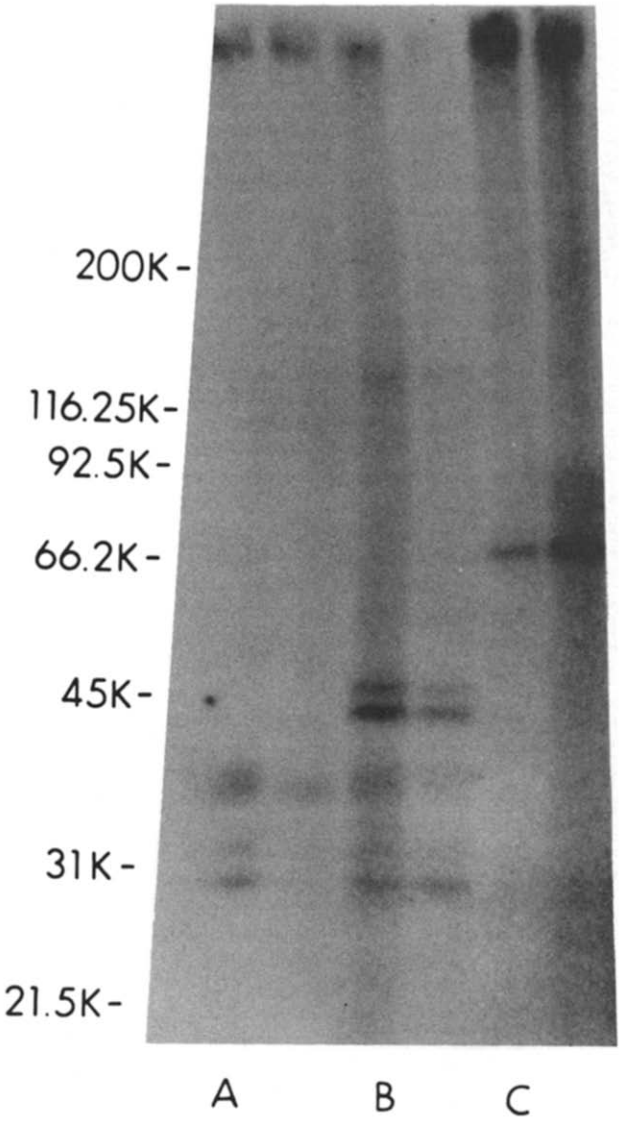

Fig. 2. SDS-PAGE electrophoresis of rat mast cell granules. (A) intact membrane granules (2.0×10^6 mast cell equivalents); (B) broken membrane granules (2.0×10^6 mast cell equivalents); and (C) 10 ml of cytosol fraction. Duplicate lanes are shown.

(see above). The failure to observe more marked labeling in the lysed granules is probably due to incomplete granule lysis, although the possibility that some of the phosphorylating activity is due to a cytoplasmic cAMP-dependent protein kinase that is firmly bound and not removed by washing is difficult to exclude.

It is apparent that mast cell granules with broken membranes phosphorylate themselves, suggesting an endogenous protein-phosphorylation activity within the granule.

Futher evaluation for possible cytoplasmic contamination

Extensive washing of the broken granules did not affect significantly the incorporation of ^{32}P into the 44K band. Incubation of areas of the gradient containing cytosol but not granules with [γ-^{32}P]ATP and Mg^{2+} yielded a 72K radioactive band as the only major radioactive band (Fig. 2). The addition of various amounts of cytosol to broken or intact granules did not increase the response in the 44K protein or cause a response to appear with intact granules. Taking these observations together with the results on lysed granules described above, the incorporation does not appear to be due to contaminating cytoplasmic proteins.

Evaluation of intact granules for protein kinase activity with exogenous substrate

The possibility was also considered that hypotonic lysis of intact granules might expose 44K protein substrate present inside the granule to a protein kinase on the granule surface. However, attempts to demonstrate protein kinase activity on intact granules with a histone substrate under conditions that

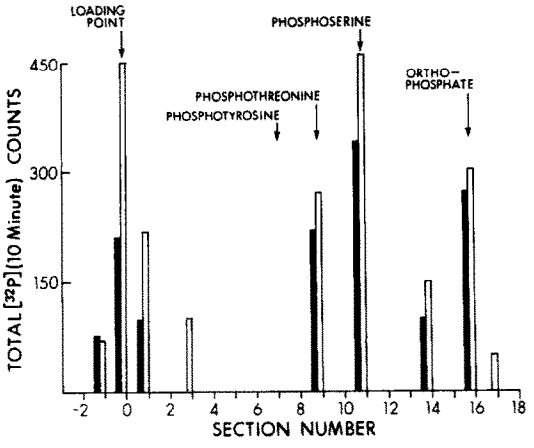

Fig. 3. Phosphoamino acid analysis of the 44K phosphoprotein. The 44K ^{32}P-labeled protein was eluted from SDS gels, precipitated with TCA, washed, and hydrolyzed in 6 N HCl at 100° for 3 hr; the hydrolysate was studied by high voltage electrophoresis on thin-layer plates in the presence of unlabeled phosphoamino acid standards as described in the text. Standards were detected by staining with ninhydrin. The TLC plates were cut into 1-cm sections (except for sections 10 and −2 where 0.5-cm sections were taken); 21 sections were taken, 18 towards the anode, 1 at the loading point (numbered 0) and 2 toward the cathode (− numbers) which were then counted for [^{32}P]-radioactivity. Where no bars are present, no radioactivity was detected. Data are shown as solid or open bars for two separate but otherwise identical experiments.

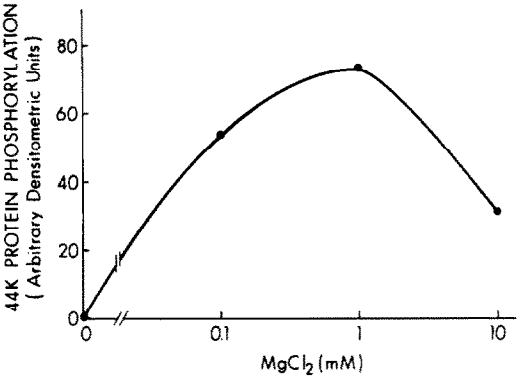

Fig. 4. Effect of $MgCl_2$ on phosphorylation of the 44K protein in rat mast cell granules. Apart from the variable amounts of $MgCl_2$, the basic incubation system and analytical gel separation systems given under Materials and Methods were used. The data represent the mean of duplicate values (within 3.2% differences).

readily demonstrate protein kinases in the cytosol [9] were unsuccessful. Granules were assayed for kinase activity with lysine-rich histones as described in Materials and Methods under conditions which give up to 8,000 cpm incorporated into histone within our usual assays with unpurified or partially purified cytosol preparations from RBL-1 cells and up to 50,000 cpm incorporated with highly active kinase preparations from other tissues (as compared with background incorporations averaging 400 cpm). No evidence for kinase activity on intact granules was obtained.

Mg^{2+} *requirements for 44K protein phosphorylation in broken membrane granules*

The effect of $MgCl_2$ on the protein phosphorylation is shown in Fig. 4. In the presence of 2 mM EDTA with $MgCl_2$ absent, the ^{32}P incorporation into the 44K band was inhibited completely. The protein phosphorylation was completely dependent on $MgCl_2$; 1 mM $MgCl_2$ produced a maximal response.

Effect of protease inhibitors on ^{32}P*-incorporation into 44K protein of broken membrane granules*

Rat mast cell granules contain considerable protease activity [15]. The effects of the protease inhibitors, DFP and PMSF, both separately and in combination, on the incorporation of ^{32}P into the 44K band of granules with broken membranes were investigated. Granules were preincubated with 2 mM DFP, 2 mM PMSF, or both for 1 hr at 30° and phosphorylated with [γ-^{32}P]ATP in the presence of inhibitors for 5 min. No effect of DFP and PMSF on the ^{32}P incorporation response was observed (data not shown).

Rate of 44K protein phosphorylation in broken membrane granules

The early time course of the protein phosphorylation is shown in Fig. 5. The incorporation of ^{32}P increased with time for 5 min and then decreased. The rate of increase of ^{32}P incorporation decreased with time, particularly after 1 min.

Effect of temperature on 44K protein phosphorylation

The temperature dependency for the protein phosphorylation is shown in Fig. 6. The maximal response was seen at 30°.

Effects of cyclic nucleotides on 44K protein phosphorylation

Cyclic GMP (from 5×10^{-5} to 5×10^{-8} M) had no effect on the protein phosphorylation (data not shown). Cyclic AMP at concentrations of 5×10^{-8}

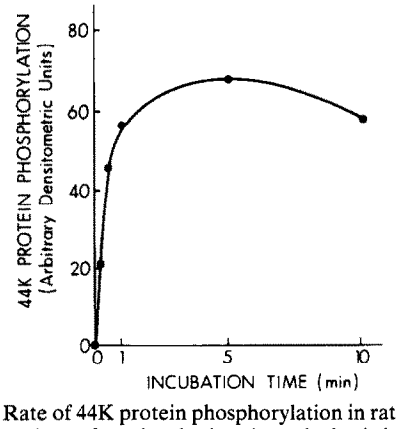

Fig. 5. Rate of 44K protein phosphorylation in rat mast cell granules. Apart from incubation time, the basic incubation and analytical systems given under Materials and Methods were used. The data represent the mean of duplicate values (within 5.9% differences).

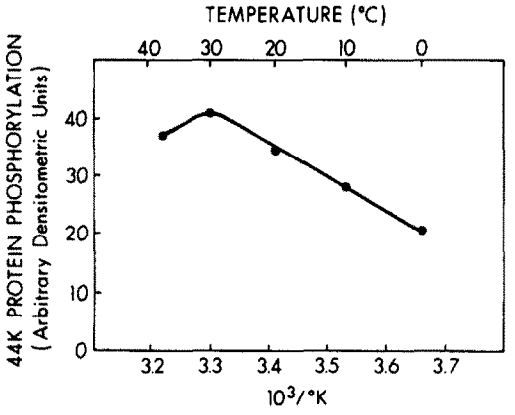

Fig. 6. Effect of temperature on phosphorylation of the 44K protein in rat mast cell granules. Apart from temperaure, the basic incubation and analytical systems given under Materials and Methods were used. The data represent the mean of duplicate values (within 2.4% differences).

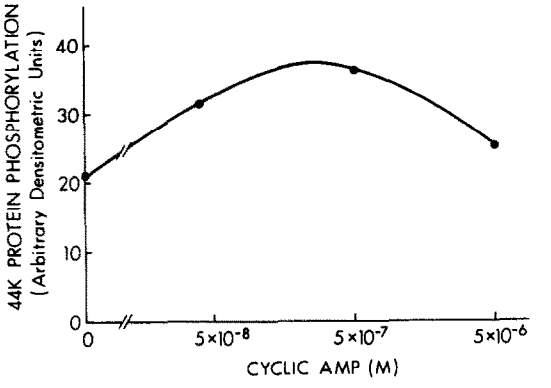

Fig. 7. Effect of cyclic AMP on phosphorylation of the 44K protein in rat mast cell granules. Apart from the variable concentrations of cyclic AMP, the basic incubation and analytical systems given under Materials and Methods were used. The data represent the mean of duplicate values (within 5.1% differences).

and 5×10^{-7} M substantially increased ^{32}P-incorporation into the 44K band (Fig. 7).

Effects of phosphatidylserine, Ca^{2+} and TPA on 44K protein phosphorylation

Studies on broken mast cells have shown that these cells contain a calcium-activated, phospholipid (phosphatidylserine and diolein)-dependent protein kinase [9]. Moreover, the calcium-dependent enzyme in rat brain is stimulated by the tumor-promoting phorbol diester TPA [16], which also promotes histamine release from human basophils [17]. For these reasons, the possible role of calcium and these various lipid activators in the 44K protein phosphorylation response was studied. Since TPA has low solubility in water, it was dissolved in DMSO and diluted to a final DMSO concentration of 0.01% (v/v). No concentration of Ca^{2+} was stimulatory, and at high Ca^{2+} concentrations (more than 2 mM) some decrease was detectable. There were no obvious effects of 60 μg)ml phosphatidylserine or 100 ng/ml TPA in either the presence or absence of Ca^{2+}. In the presence of 0.5 mM $CaCl_2$, 5 mM EGTA obviously enhanced ^{32}P-incorporation (Fig. 8). It is apparent that Ca^{2+} inhibits rather than stimulates protein phosphorylation in this system, suggesting strongly that a calcium-dependent protein kinase is not involved.

Two-dimensional SDS/polyacrylamide-gel electrophoresis of labeled granules with broken membranes in the presence or absence of 5 mM EGTA demonstrated that the 44K phosphoprotein is made up of several components differing in pI from approximately 7.6 to 6.6 (Fig. 9) (uncorrected for possible effects of urea on electrophoretic behavior in isoelectric focusing gels).

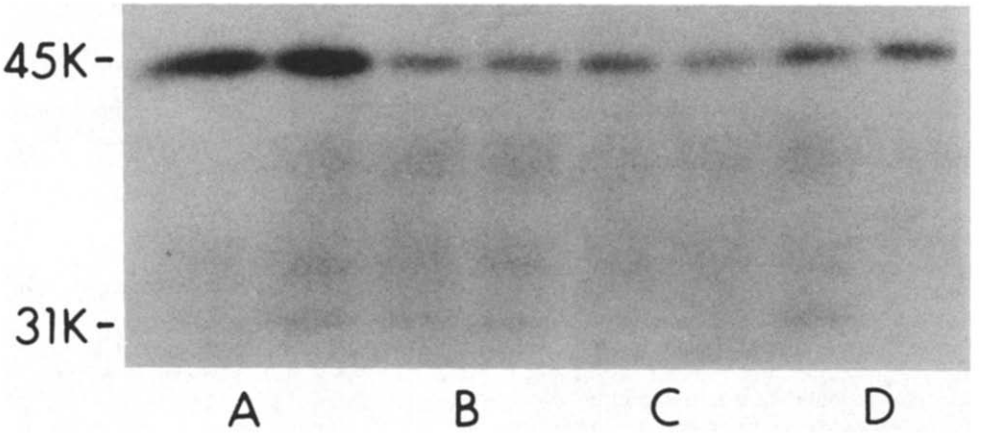

Fig. 8. Autoradiographs of one-dimensional SDS/polyacrylamide gels of granules with broken membranes (2.6×10^6 mast cell equivalents) labeled with [γ-^{32}P]ATP in the presence and absence of EGTA. (A) 0.5 mM Ca^{2+} with 5 mM EGTA; (B) 0.5 mM Ca^{2+} without EGTA; (C) 0.5 mM Ca^{2+} with 60 μg/ml phosphatidylserine; and (D) 0.5 mM Ca^{2+} with 100 ng/ml TPA.

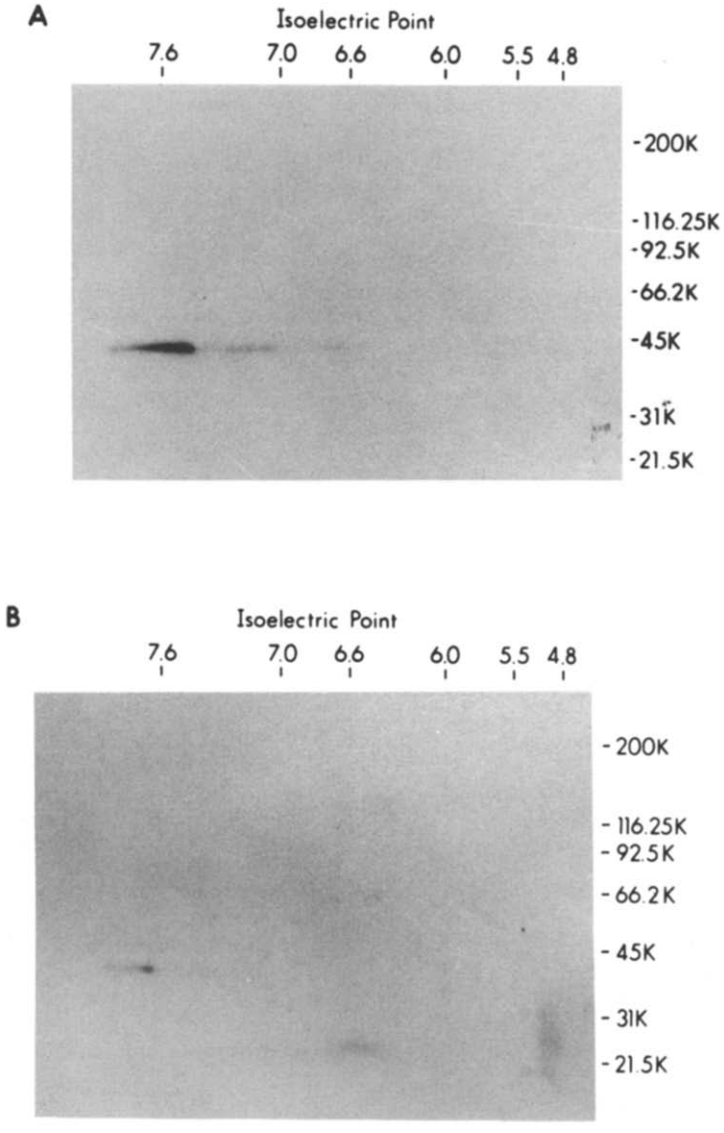

Fig. 9. Autoradiographs of granules with broken membranes (1.8×10^6 mast cell equivalents) in two-dimensional SDS/polyacrylamide gels after incubation with [γ-^{32}P]ATP and Mg^{2+} in the presence and absence of EGTA. (A) With 5 mM EGTA during labeling; and (B) without EGTA.

DISCUSSION

We have shown in this report that a single polypeptide with a molecular weight of 44,000 daltons was phosphorylated when broken rat mast cell granules were incubated with [γ-^{32}P]ATP and Mg^{2+}. Acid hydrolysis showed that [^{32}P]phosphate radioactivity in the granule protein was present primarily as phosphoserine and phosphothreonine, suggesting strongly that the phosphate transfer reaction was catalyzed by a protein kinase. In contrast to broken granules, intact granules failed to show the phosphorylation response, even in autoradiographs with a relatively long exposure time. However, when the intact granules were lysed in hypotonic medium before incubation with [γ-^{32}P]ATP, the 44K phosphorylated protein band was seen again. Taken together, these observations suggest strongly that a phosphorylation system is present at the inside of the granule membrane. More phosphorylating activity was present when broken granules were isolated directly from the cell than from intact granules which were subsequently broken. This was probably due to incomplete granule lysis, but the possibility has to be considered that a portion of the phosphorylating activity was derived from the cytosol. In any case our evidence indicates that a cAMP-dependent protein kinase can associate firmly with granules where it could be important either in the release of granules from the cell or in their later reconstitution to intact granules.

The initial phosphorylation reaction was rapid and maximal at 30°. After 5 min, the incorporated radioactivity decreased slightly. Possible explanations for

the marked decrease in rate of phosphate uptake with time include hydrolysis of ATP, partial utilization of protein substrate, dephosphorylation by a phosphorprotein phosphatase [18, 19] or proteolytic degradation of the phosphorylating enzyme or substrate. The last possibility is unlikely to be a major factor since protease inhibitors such as DFP and PMSF did not enhance phosphorylation. However, even though no obvious effect of protease inhibitors such as DFP or PMSF on overall phosphorylation was observed, degradation of overall phosphorprotein after phosphorylation by a protease which is not affected by these inhibitors is not completely excluded.

The protein phosphorylation reaction was enhanced by cyclic AMP (50–500 nM), unaffected by cyclic GMP, and inhibited by Ca^{2+} both in the presence and absence of lipids that enhance the activities of calcium-dependent protein kinases. Taken together, these observations suggest strongly that the protein kinase responsible for the phosphorylation was a cyclic AMP dependent protein kinase. Mast cells have been shown previously to have a high cyclic AMP content by radioimmunoassay [20, 21] which rapidly increases after activation of mediator release by antigen and IgE or concanavalin A. Immunofluorescence studies with anti-cyclic-AMP antibodies suggest that mast cell granules contain substantial amounts of bound cyclic AMP [20]. Since granules generally contain ATP [22], the cyclic AMP generating and degrading enzymes also may be present, providing a complete system for regulating the cyclic AMP dependent protein kinase inside the granule. However, as far as we are aware, direct measurements of ATP, adenylate cyclase and cyclic AMP in isolated mast cell granules have not yet been made and the source and location of the bound cyclic AMP seen by immunofluorescence remain to be established. Regulation of the granule kinase in intact cells may involve cyclic AMP derived entirely from the plasma membrane. In this event the cyclic AMP would probably only be available to the enzyme when the granule membranes underwent lysis and fusion.

The nature of the substrate for the 44K protein phosphorylation is uncertain. Components of cyclic AMP dependent protein kinases are phosphorylated [23–26], including the catalytic subunit (M_r approximately 40K) of rabbit skeletal muscle cyclic AMP dependent protein kinase [26]. Studies in progress indicate that at least one type of cAMP dependent protein kinase in RBL-1 cells contains a 44K autophosphorylated component, and we suspect that a similar protein is responsible for the phosphorylizing activity in broken granules from mast cells. Platelets, the secretory responses of which have many similarities to those of mast cells, contain a 40K protein which is phosphorylated in intact platelets in response to thrombin [27]. This protein was originally reported to be phosphorylated by a cyclic AMP dependent protein kinase in broken platelet preparations [27]. A probably identical protein with an apparent molecular weight of 47K which is phosphorylated in platelets and has a pI (6.1–6.5, after phosphorylation) somewhat similar to our mast cell 44K phosphorylated protein (6.6–7.6) has been described by Imaoka *et al.* in thrombin and collagen activated platelets [28]. However, according to recent reports [29, 30] the 40–47K protein in platelets is phosphorylated by a calcium-dependent protein kinase in direct contrast to the 44K phosphoprotein in mast cells.

Regardless of the precise nature of the protein substrate and its relationship to the phosphorylated protein in platelets, many physiological reactions are regulated by protein phosphorylation, and it is possible that the endogenous protein phosphorylation system in mast cell granules is involved in the control of granule secretion. In this connection there is evidence that when purified mast cells are activated by anti-rat F $(ab')_2$ or antigen, serial changes occur in the phosphorylating activity of the cytosol for exogenous substrates in the presence or absence of cyclic AMP [8, 31]. These observations suggest that a cyclic AMP dependent enzyme is being activated intracellularly. While our studies with granules indicate that the protein kinase inside the granules is also activated by cyclic AMP, the protein kinase involved and even the source of cyclic AMP may well be different. Careful comparison of the kinetics of phosphorylation of cytosol proteins and the 44K protein in intact cells will be of interest and may help to clarify the mechanism and significance of the granule protein phosphorylation.

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