Antigen-induced Secretion of Histamine and the Phosphorylation of Myosin by Protein Kinase C in Rat Basophilic Leukemia Cells*

(Received for publication, December 14, 1988)

Russell I. Ludowyke‡, Itzhak Peleg‡, Michael A. Beaven‡, and Robert S. Adelstein‡

From the ‡Laboratory of Chemical Pharmacology and the §Laboratory of Molecular Cardiology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

IgE-mediated stimulation of rat basophilic leukemia (RBL-2H3) cells results in the secretion of histamine. Myosin immunoprecipitated from these cells shows an increase in the amount of radioactive phosphate incorporated into its heavy (200 kDa) and light (20 kDa) chains. In unstimulated cells, two-dimensional mapping of tryptic peptides of the myosin light chain reveals one phosphopeptide containing the serine residue phosphorylated by myosin light chain kinase. Following stimulation a second phosphopeptide appears containing a serine residue phosphorylated by protein kinase C. Tryptic phosphopeptide maps derived from myosin heavy chains show that unstimulated cells contain three major phosphopeptides. Following stimulation a new tryptic phosphopeptide appears containing a serine site phosphorylated by protein kinase C.

The stoichiometry of phosphorylation of the myosin light and heavy chains was determined before and after antigenic stimulation. Before stimulation, myosin light chains contained 0.4 mol of phosphate/mol of light chain all confined to a serine not phosphorylated by protein kinase C. Cells that secreted 44% of their total histamine in 10 min exhibited an increase in phosphate content at sites phosphorylated by protein kinase C from 0 mol of phosphate/mol of myosin subunit to 0.7 mol of phosphate/mol of light chain and to 1 mol of phosphate/mol of heavy chain. When RBL-2H3 cells were made permeable with streptolysin O they still showed a qualitatively similar pattern of secretion and phosphorylation. Our results show that the time course of histamine secretion from stimulated RBL-2H3 cells parallels that of myosin heavy and light chain phosphorylation by protein kinase C.

The two major contractile proteins, actin and myosin, play an important role in the contraction of skeletal, cardiac, and smooth muscle as well as in a variety of cellular functions in nonmuscle cells. These include cell movement, shape change, and cytokinesis (1-3). Contractile activity results from the sliding of myosin and actin filaments past one another, with ATP supplying the energy necessary for this physical movement. The ability of myosin to hydrolyze ATP under the stimulus of actin (the actin-activated MgATPase activity of myosin) effects this chemical to mechanical transformation. For vertebrate smooth muscle and nonmuscle cells it has been shown that the in vitro actin-activated MgATPase activity of myosin can be regulated by phosphorylation of the 20-kDa myosin light chain, a reaction catalyzed by a Ca2+-calmodulin-dependent enzyme, myosin light chain kinase (MLCK). 1 Studies with "skinned" smooth muscle fibers as well as intact strips of smooth muscle have confirmed the importance of this phosphorylation in initiating contractile activity (1-4). In nonmuscle cells such as platelets, myosin light chain phosphorylation has been implicated in shape change and granule secretion (5-7).

In addition to being a substrate for MLCK, the 20-kDa light chain of smooth muscle and platelet myosin can be phosphorylated by protein kinase C (8-10). The effect of this phosphorylation in vitro is to decrease the actin-activated MgATPase activity of myosin that has been phosphorylated by MLCK (8, 10). Moreover, if myosin is first phosphorylated by protein kinase C, it becomes a much poorer substrate for MLCK than the unphosphorylated form (8). However, the physiologic role of this phosphorylation is as yet unclear.

A major attribute of the rat basophilic leukemia cell line, RBL-2H3, a cultured analog of rat mucosal mast cells (11, 12), is that exocytosis can be readily quantitated by measurement of the secretion of histamine or [3H]5-hydroxytryptamine ([3H]5-HT). These cells contain receptors for IgE, and multivalent binding of antigen, such as dinitrophenylated bovine serum albumin (DNP-BSA) to receptor-associated IgE, results in the release of histamine as well as a variety of other chemical mediators of inflammation. The time courses of secretion and of the intervening signals that are thought to promote this secretion have been well characterized in these cells. The early events include antigen-induced aggregation of receptors for IgE (12), rapid hydrolysis of inositol phospholipids via the activation of a GTP-binding protein, elevation of [Ca2+]i; (13), translocation of protein kinase C to the plasma membrane (14), and phosphorylation of diverse proteins (15). All of these events correlate in extent with the amount of antigen added and with the extent of the secretory response. Furthermore, all experimental manipulations that cause a decrease in hydrolysis of inositol phospholipids cause an equal reduction in the rise in [Ca2+]i, and secretion of histamine (13, 16). These observations are consistent with the idea that the antigen-induced hydrolysis of inositol phospholipids generates synergistic signals for secretion, namely the mobilization of Ca2+ via the production of inositol 1,4,5-trisphosphate and the activation of protein kinase C by di...

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: MLCK, myosin light chain kinase; 5-HT, 5-hydroxytryptamine, serotonin; [Ca2+]i, concentration of cytosolic Ca2+; DNP, dinitrophenol; BSA, bovine serum albumin; DNP-BSA, antigen which contained 24 molecules of dinitrophenol conjugated with 1 molecule of bovine serum albumin; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); CHAPS, 3-(3-cholamidopropyl)dimethylammonio)1-propanesulfonate; TEMED, N,N,N',N"-tetramethylethylenediamine; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; IEF, isoelectric focusing; GTPγS, guanosine 5'- (3-O-thio) triphosphate.
acylglycerol (17, 18) although other unidentified signals may also be generated (19).

RBL-2H3 cells have other attributes that make them particularly useful for studying the function of myosin in non-muscle cells. Following antigen stimulation, these cells undergo mechanical changes which include extensive ruffling of the plasma membrane, fusion of perigranular and plasma membranes, and finally the exocytotic release of granule constituents (12, 20). Whether contractile proteins participate in the process of exocytosis in these cells is unknown at present.

Studies of the in situ phosphorylation of cellular proteins using radioactive phosphate can be ambiguous if the labeling of proteins does not distinguish between a net increase in the amount of phosphate and an increase in radioactive phosphate turnover. Moreover, it is always useful to know the stoichiometry of phosphorylation when trying to relate a cellular event, such as release of histamine, to a change in protein phosphorylation, since very low levels of phosphorylation diminish the likelihood of a causal relationship (for review see Ref. 21).

The present study reveals that histamine release from RBL-2H3 cells is accompanied by an increase in phosphorylation of the 20-kDa myosin light chain and the 200-kDa myosin heavy chain. Both phosphorylations appear to be catalyzed by protein kinase C. We also quantitate this phosphorylation and show that the time course and extent of protein kinase C phosphorylation of myosin heavy chains and light chains are correlated with histamine secretion. Furthermore, when RBL-2H3 cells are permeabilized with streptolysin O (22), they still show the same pattern of antigen-mediated secretion and phosphorylation of myosin. While the findings do not establish a causal relationship between myosin phosphorylation and secretion, the present work provides the first indication that such a relationship might exist.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**RBL-2H3 cells were maintained as monolayer cultures and harvested by trypsin treatment as described previously (11, 23, 24). Cells were harvested from monolayer cultures and transferred to 60-mm Petri dishes at a density of 2.5  10^6 cells in 3 ml of Eagle’s modified Eagle’s medium supplemented with 15% fetal bovine serum (GIBCO). The cells were sensitized with DNP-specific IgE by the addition of 0.5 mg/ml IgE and incubated overnight at 37 °C with 5% CO2. The cells were then washed twice in a phosphate-buffered saline, and the washed pellet was recovered by sedimentation for 2 min in an Eppendorf microcentrifuge. To aspirate the residual buffer and placing the dishes on ice.

**Preparation of Cell Extracts and Immunoprecipitation—**Following stimulation and aspiration, the cells were immediately disrupted by scraping them in 0.6 ml of “lysis” buffer that contained 1% Nonidet P-40, 100 mM sodium pyrophosphate, 250 mM NaCl, 50 mM NaF, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 15 mM β-mercaptoethanol, and 20 mM Tris-HCl (pH 7.9). The lysate was sedimented at 100,000 × g for 5 min at 4 °C, and the supernatant fraction (0.5 ml containing 1-2 mg/ml protein) was used as the pellet myosin antibody, IgG fraction (20 mg/ml) for 1 h at 4 °C with shaking. The characteristics of this antibody have been described (26). Ten times the antibody volume of a standardized *Staphylococcus aureus* cell suspension (Pansorbin, Calbiochem) was added for an additional 1 h of incubation, and the mixture was sedimented for 2 min in an Eppendorf microcentrifuge. The pellet was washed with 0.5 ml of the lysis buffer followed by a second wash with 0.5 ml of the same buffer, which was diluted 50% by volume with phosphate-buffered saline, and the washed pellet was dissolved in 100 μl of SDS sample buffer (27). Gel electrophoresis was performed in 0.1% SDS-12.5% polyacrylamide using the buffer system of Laemmli (27). Autoradiography was performed using Kodak XAR-x-ray film and a Kodak X-Omat M20 processor. Coomassie Blue-stained gels and autoradiograms were scanned using a laser densitometer (LKB Ultrascan XL) to quantitate the relative amounts of proteins and radioactivity.

**Measurement of Histamine Secretion—**The release of histamine from RBL-2H3 cells was measured by ion-sensitive microphotonics (that such a relationship might exist).

**Preparation of Samples—**Two-dimension gel electrophoresis—Immunoprecipitated samples of myosin were analyzed by two-dimension polyacrylamide gel electrophoresis as described by O’Farrell (28) but with modifications. Following the first wash of the Pansorbin-antibody-myosin immunoprecipitate with lysis buffer, the pellet was suspended in 0.5 ml of Ringer’s buffer. This fraction was then divided into two parts (usually 1/5 and 4/5). Pellets were recovered by sedimentation for 2 min in an Eppendorf centrifuge. To

**Phosphoamino Acid Analysis—**The eluted peptides were subjected to acid hydrolysis in 6 ml of 6 N HCl for 3 h at 100 °C in vacuo. The resultant hydrolysate was lyophilized to dryness, resuspended in 10 μl of electrophoresis buffer containing acetic acid:formic acid:water (15:5:80) before digestion to a solution containing n-butyryl alcoholsphoropeptide-acid H2O (195:150:30:120) for myosin light chains and n-butyryl alcoholsphoropeptide-acid H2O (127.5:22.5:45:15:90) for heavy chains. The plates were dried and then autoradiographed as described above. Radioactive peptides were scraped from the plates for assay of radioactivity or, after elution with 1 ml of 50 mM NH4HCO3, for phosphoamino acid analysis.

**Preparation of Cell Extracts and Immunoprecipitation—**Following stimulation and aspiration, the cells were disrupted by
quantitate the radioactive phosphate content of the myosin heavy chains and light chains, the smaller pellet was resuspended in 50 μl of SDS buffer and subjected to electrophoresis in 0.1% SDS-12.5% polyacrylamide gels (27). The larger pellet was resuspended in 200 μl of an IEF buffer that contained 9.5 M urea, 4% Nonidet P-40, 2% ampholytes (pH 4–6, Bio-Lyte, Bio-Rad, and 0.1 M diethanol (recipe solution X, 1.27%), and 0.28 ml of a CHAPS/Nonidet P-40 solution (0.1 g CHAPS, 0.3 ml of 0.5 M Tris-HCl, pH 6.8, 0.5 ml of 10% SDS, and 0.5 ml of deionized water, and 0.5 ml of Nonidet P-40) were added and allowed to polymerize the polyacrylamide.

The anolyte contained 4 mM phosphoric acid and the catholyte contained 20 mM NaN3. After adding the latter solution to the upper chamber, excess unpolymerized gel solution was removed from the top of the gel prior to addition of the sample (up to 40 μl). IEF was carried out at room temperature with a constant voltage of 200 V for 2 h, followed by 500 V for 5 h, and then overnight at 2,000–3,000 V to achieve an additional 32,000 V-h.

**Second Dimension SDS-PAGE**—The system used was the Hoeffer model SE600. Sufficient material for two separating gels (160 × 120 × 1.5 mm) was prepared as 15% polyacrylamide/4% bisacrylamide (40 ml of polyacrylamide/bisacrylamide (30%/0.8%), 30 ml of 1.5 M Tris-HCl, pH 8.8, 0.5 ml of 10% SDS, and 9.2 ml of deionized water. The solution was degassed and polymerization initiated with 0.06 ml of TEMED and 0.5 ml of 10% ammonium persulfate. Stacking gels (1.5 mm) of 5% polyacrylamide/0.13% bisacrylamide were prepared with 2.36 ml of polyacrylamide/bisacrylamide (30%/0.8%), 0.5 ml of 0.5 M Tris-HCl, pH 6.8, 0.2 ml of 10% SDS, and 1.15 ml of deionized water. After degassing, appropriate concentrations of TEMED and ammonium persulfate were added as above. After separation in the first dimension, the IEF gels were removed from the glass tubes and placed on a sheet of Parafilm. The gels were trimmed to a length of 10 cm from the acidic end (i.e., the bottom) of the gel to fit over the second dimension gel. A small volume of 1 hot agar solution (0.5% in SDS sample buffer without bromphenol blue) was then placed into the slot above the stacking gel. The IEF gel was then rinsed with 350 μl of a transfer solution (200 mM NaCl, 0.1% SDS, 0.025% Triton X-100, and 0.5 M Tris-HCl, pH 6.8, 5.14 ml of deionized water, and 0.5 ml of 0.5% Triton X-100). The IEF gel was then quickly placed in the agar solution above the second dimension gel and sealed with more agar. The gels were run at room temperature at a constant voltage of 200 V for 5–6 h following which they were stained with Coomassie Blue. Since there was not enough RBL-2H3 myosin light chain for identification by staining, the 20- and 17-kDa light chains of turkey gizzard myosin (kindly supplied by Dr. J. R. Sellers, National Heart, Lung, and Blood Institute) were added before IEF to serve as standards. Interestingly, while the 17-kDa gizzard myosin light chains and the equivalent RBL-2H3 myosin light chain coelectrophoresed, the 20-kDa gizzard myosin light chains focused to the basic side of the equivalent RBL-2H3 myosin light chains.

Gels containing 35S-labeled proteins were destained before immersion for 30 min in Enlightning solution (Du Pont-New England Nuclear Research Products) and then dried on Whatman no. 3MM filter paper in a heated vacuum dryer. Gels containing 32P-labeled proteins were similarly between cellulose sheets (Bio-Rad). Gels were then subjected to autoradiography and the resultant x-ray film scanned as described above.

**Isotopic Quantitation by Radioactive Phosphate**—Following autoradiography, the dried gel was placed on Whatman no. 3MM filter paper in a heat vacuum dryer. Gels were then subjected to autoradiography and the resultant x-ray film scanned as described above.

**Identification of the Sites Phosphorylated in the 20-kDa Myosin Light Chain**—Previous work has demonstrated that the 20-kDa myosin light chain of gizzard smooth muscle myosin (9,10) and platelet myosin (34) contains five different sites of phosphorylation. Two of these, serine 19 and threonine 18 in gizzard myosin, were phosphorylated by MLCK and three, serine 2 and threonine 9, by protein kinase C (9,10). To identify the sites of myosin light chain phosphorylation in control and DNP-BSA-stimulated cells, we carried out two-dimensional peptide mapping of tryptic digests of the 20-kDa light chains. Fig. 2 shows autoradiograms of these maps. In unstimulated cells (control), 32P is confined to a single major phosphopeptide (Fig. 2, Control, spot 1, which appears as a doublet that comigrates with the phosphopeptide that was generated in vitro following phosphorylation of human platelet myosin light chains by MLCK (data not shown). Phosphoamino acid analysis of this peptide reveals the presence of phosphoserine alone, confirming that it represents the phosphoserine that was detected in the autoradiograms.}

**Results**

**Time Course of Myosin Phosphorylation**—Fig. 1A is a representative autoradiogram of 32P-labeled myosin to illustrate the time course of myosin heavy chain and light chain phosphorylation. RBL-2H3 cells were stimulated with DNP-BSA, the reaction was terminated at the indicated times, and the myosin was recovered selectively by immunoprecipitation as described under “Experimental Procedures.” The figure shows that both the 200-kDa myosin heavy chain and the 20-kDa myosin light chain are phosphorylated in the absence of antigen and that phosphorylation of these peptides did not increase with time (Fig. 1A, Antigen). Following antigen stimulation there appears to be an increase in the phosphorylation of both myosin heavy and light chains (Fig. 1A, + Antigen).

Fig. 1B is a graph constructed from a scan of the autoradiogram shown in Fig. 1A. The data indicate an apparent increase in radioactive phosphate associated with the myosin heavy and light chains in stimulated cells and show that maximal phosphorylation is observed 5–10 min after addition of DNP-BSA. Thereafter there is a decline in the amount of radioactive phosphate associated with both heavy and light chains to values approaching that found in unstimulated cells. Fig. 1B also shows that the time course of histamine secretion from stimulated cells parallels that of myosin phosphorylation. Histamine accumulation in the medium approaches a maximum at 10 min. Thereafter only a small additional amount of histamine is released.

**Identification of the Sites Phosphorylated in the 20-kDa Myosin Light Chain**—Previous work has demonstrated that the 20-kDa myosin light chain of gizzard smooth muscle myosin (9,10) and platelet myosin (34) contains five different sites of phosphorylation. Two of these, serine 19 and threonine 18 in gizzard myosin, were phosphorylated by MLCK and three, serine 2 and threonine 9, by protein kinase C (9,10). To identify the sites of myosin light chain phosphorylation in control and DNP-BSA-stimulated cells, we carried out two-dimensional peptide mapping of tryptic digests of the 20-kDa light chains. Fig. 2 shows autoradiograms of these maps. In unstimulated cells (control), 32P is confined to a single major phosphopeptide (Fig. 2, Control, spot 1, which appears as a doublet that comigrates with the phosphopeptide that was generated in vitro following phosphorylation of human platelet myosin light chains by MLCK (data not shown). Phosphoamino acid analysis of this peptide reveals the presence of phosphoserine alone, confirming that it represents the phosphoserine that was detected in the autoradiograms.
Myosin Phosphorylation and Histamine Secretion

12495

A B

MYOSIN
HEAVY CHAIN-

LIGHT CHAIN-

DYE
FRONT-

15'  1'  2.5'  5'  10'  20'

2.6
wm

FIG. 1. Autoradiogram and graph indicating the time course of phosphorylation of myosin heavy and light chains and histamine release. A, cells were labeled with [32P]orthophosphoric acid for 2-3 h and stimulated with the antigen DNP-BSA for 15 s to 30 min as indicated (+ Antigen). The myosin was immunoprecipitated with anti-platelet myosin (IgG fraction) and analyzed by SDS-PAGE using 12.5% polyacrylamide gels which were subjected to autoradiography. Control (- Antigen) cells were treated the same as the stimulated cells, but buffer was added instead of DNP-BSA for the times indicated. B, the amount of histamine released at each time point was calculated as a percent of total histamine as described previously (25). The extent of phosphorylation of myosin heavy and light chains was determined after correction for protein loading (by scanning the Coomassie Blue-stained myosin heavy chain) by scanning the autoradiogram shown in A using a laser densitometer. These data are a representative set from five different experiments.

Control

1 Min

10 Min

30 Min

FIG. 2. Autoradiograms of two-dimensional peptide maps of tryptic digests of 32P-labeled myosin light chains. Myosin was immunoprecipitated from control cells and cells stimulated with DNP-BSA for the indicated times, analyzed on SDS-PAGE (12.5% polyacrylamide), and the light chains were subjected to tryptic digestion and peptide mapping as described under "Experimental Procedures." The numbers indicate the phosphopeptides that comigrated with phosphopeptides obtained from human platelet and turkey gizzard myosin light chains which were phosphorylated in vitro by MLCK (1) and protein kinase C (2). E indicates direction of electrophoresis toward the anode and C indicates direction of chromatography.

Corresponds to serine 19 in turkey gizzard myosin light chains. Fig. 2 also shows that 1 min following stimulation by DNP-BSA a second major phosphopeptide appears (1 Min, spot 2). This peptide comigrates with tryptic peptides derived from smooth muscle and platelet myosin light chains that were known to be phosphorylated in vitro by protein kinase C (9, 10, 34). Phosphoamino acid analysis reveals the presence of phosphoserine confirming that the site phosphorylated was serine 1 or 2.

Two-dimensional Peptide Mapping of the Tryptic Peptides from 32P-Labeled Myosin Heavy Chain—Fig. 1 showed an increase in 32P associated with the myosin heavy chain following DNP-BSA stimulation. It was of interest to determine whether this apparent increase in heavy chain phosphorylation was confined to a specific site on the myosin heavy chain. Fig. 3 shows autoradiograms of two-dimensional tryptic peptide maps of the RBL-2H3 cell myosin heavy chain in unstimulated cells (Control) and following 10 min of stimulation by DNP-BSA. In unstimulated cells three major myosin tryptic phosphopeptides are present. Following stimulation, a single new tryptic phosphopeptide appears (Fig. 3, Stimulated (10'), marked with an arrow). Phosphoamino acid analysis showed that this newly phosphorylated peptide contained phosphoserine. The appearance of this phosphopeptide coincides in time with the appearance of phosphopeptide 2 (see Fig. 2) from the myosin light chain. The time course of phosphorylation of the myosin heavy chain parallels that of the myosin light chain, reaching a maximum at about 10 min and declining toward the levels in unstimulated cells by 30 min (see Fig.
No other new myosin heavy chain tryptic phosphopeptide was observed during this period nor was the intensity of the three original tryptic phosphopeptides shown in the control panel of Fig. 3 altered (compare the Control and Stimulated panels).

To confirm that this de novo phosphorylation of the myosin heavy chain was most likely due to protein kinase C, we ran a separate peptide map of the tryptic peptides of human platelet myosin heavy chain (Fig. 3, Platelets) that had been phosphorylated by protein kinase C in vitro (34). Two-dimensional peptide mapping showed that these two peptides have similar mobilities. This was confirmed by comigration of the two peptides (data not shown) and reinforces the idea that the increase in myosin heavy chain phosphorylation found in these antigen-stimulated cells is due to activation of protein kinase C.

Separation of Unphosphorylated and Phosphorylated Forms of Myosin Light Chains by Two-dimensional Gel Electrophoresis—Fig. 4 shows autoradiograms depicting the separation of the unphosphorylated and phosphorylated forms of the 20-kDa myosin light chains that were immunoprecipitated from cells labeled with either [3S]methionine or [32P]orthophosphoric acid. Separation was by two-dimensional gel electrophoresis with IEF (pH 4-6) in the first dimension and SDS-PAGE in the second. The autoradiogram of the 35S-labeled myosin light chains from unstimulated (Control) cells revealed two major forms of the 20-kDa myosin light chain. One corresponded to unphosphorylated myosin light chains (U-P) and the other to monophosphorylated myosin light chains (P1). This identification was made by introducing an aliquot of turkey gizzard myosin light chains into both the 35S- and 32P-labeled samples just prior to two-dimensional gel electrophoresis as described under “Experimental Procedures.” The Coomassie Blue-stained gizzard light chains permitted alignment of the 35S-labeled myosin light chains (Fig. 4, left) with the 32P-labeled myosin light chains (Fig. 4, right) and the identification of the 35S-labeled myosin light chain (U-P) to the basic side of the light chain labeled P1 as the unphosphorylated form (confirmed by the lack of a corresponding radioactive spot on the 32P autoradiogram).

When 35S-labeled cells were stimulated for 10 min with DNP-BSA, there was an increase in the amount of mono-phosphorylated myosin light chain (P1) compared with unstimulated controls, as well as the appearance of a diphosphorylated form of the myosin light chain (P2) (Fig. 4, lower left). Correspondingly, in the 32P-labeled cells two phosphorylated forms were also seen, P1 and P2, which corresponded to mono- and diphosphorylated myosin light chains, respectively (Fig. 4, lower right). In order to carry out tryptic peptide mapping of each of these phosphorylated forms as described below, these two forms were more widely separated in the isoelectric focusing dimension than were the 35S-labeled light chains.

Two-dimensional Mapping of Tryptic Phosphopeptides from 35S-Labeled Myosin Light Chains Separated by Two-dimensional Gel Electrophoresis—In order to identify the sites of phosphorylation, the 35S-labeled myosin light chains shown in Fig. 4 were cut from the gels, digested with trypsin, and two-dimensional mapping performed as described above. The autoradiogram in Fig. 5 (upper panel) of the trypsin-digested monophosphorylated myosin light chain (P1) from unstimulated cells shows one phosphopeptide, demonstrated previously to contain the serine residue that is phosphorylated by MLCK. In contrast, tryptic digestion of the monophosphorylated light chains (P1) in the stimulated cells yielded two phosphopeptides: (Fig. 5, lower left) peptide 1, containing the residue phosphorylated by MLCK and peptide 2, containing the serine residue phosphorylated by protein kinase C.

A similar pattern of two phosphopeptides was observed following digestion of the diphosphorylated myosin light chains (Fig. 5, lower right). There is an additional minor peptide doublet seen just to the right of the origin in the
peptide 1 (the MLCK site) and 51% in peptide 2 (the protein chain and 0.12 mol by the diphosphorylated form for a total was 0.25 mol contributed by the monophosphorylated light chain). Since 50% of the myosin light chain was phosphorylated by protein kinase C. Scanning of the kinase C site). Since 49% of the radioactive phosphate was present in the monophosphorylated, the stoichiometry at the MLCK site phosphorylated by myosin light chain kinase and this established the stoichiometry of this site phosphorylated by MLCK, and this established the stoichiometry of phosphorylation of the two different sites on the myosin light chain at various intervals following addition of antigen to the cells. The mean results of five separate experiments are presented. For this set of experiments the stoichiometry of phosphorylation was determined as follows. Two-dimensional gel electrophoresis of 35S-labeled cells was first used to establish the stoichiometry of phosphorylation of the myosin light chains in control cells. Similar to the results reported above for a separate batch of cells, this was found to be approximately 0.4 mol of phosphate/mol of myosin light chain. For each experiment the immunoprecipitated myosin from all the time points was run on a single SDS-polyacrylamide gel. This permitted separation of the 20-kDa myosin light chain and standardization of the amount of myosin loaded on the gel (based on Coomassie Blue staining of the heavy chain). The light chains were subjected to two-dimensional tryptic peptide analysis which confirmed that only the myosin light chain kinase site was phosphorylated in control cells. The amount of radioactive phosphate found in this peptide was therefore be 0.38 mol of phosphate/mol of light chain (see Table II, Cell Batch 1, light chain site).

The stoichiometry of phosphorylation of the RBL-2H3 myosin heavy chain was calculated by cutting out the heavy and light chains from the same one-dimensional SDS-polyacrylamide gel and determining the radioactivity of each by Cerenkov counting. Knowing that the radioactive phosphate in the myosin light chain was equivalent to a stoichiometry of 0.41 mol of phosphate/mol of light chain in unstimulated cells, we calculated the stoichiometry of phosphorylation of myosin heavy chain in these cells as 1.04 mol of phosphate/mol of heavy chain. Following 10 min of antigenic stimulation this increased to approximately 1.61 mol of phosphate/mol of heavy chain. Above we showed that the phosphate on the heavy chain in unstimulated cells was divided among three major tryptic phosphopeptides (Fig. 3). Following antigenic stimulation, these phosphopeptides did not change in intensity, although a single new tryptic phosphopeptide containing only phosphoserine appeared. The appearance of this phosphopeptide has been demonstrated to be due to protein kinase C activation. We calculated a stoichiometry of 0.57 mol of phosphate/mol of heavy chain due to protein kinase C activation assuming that the phosphopeptide contains a single serine (see Table II).

Changes in the Stoichiometry of Phosphorylation during the Course of Stimulation—Fig. 6 shows the stoichiometry of phosphorylation of the two different sites on the myosin light chain at various intervals following addition of antigen to the cells. The mean results of five separate experiments are presented. For this set of experiments the stoichiometry of phosphorylation was determined as follows. Two-dimensional gel electrophoresis of 35S-labeled cells was first used to establish the stoichiometry of phosphorylation of the myosin light chains in control cells. Similar to the results reported above for a separate batch of cells, this was found to be approximately 0.4 mol of phosphate/mol of myosin light chain. For each experiment the immunoprecipitated myosin from all the time points was run on a single SDS-polyacrylamide gel. This permitted separation of the 20-kDa myosin light chain and standardization of the amount of myosin loaded on the gel (based on Coomassie Blue staining of the heavy chain). The light chains were subjected to two-dimensional tryptic peptide analysis which confirmed that only the myosin light chain kinase site was phosphorylated in control cells. The amount of radioactive phosphate found in this peptide was therefore

<table>
<thead>
<tr>
<th>State of phosphorylation</th>
<th>Distribution</th>
<th>Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstimulated</td>
<td>Stimulated</td>
</tr>
<tr>
<td>Unphosphorylated (U-P)</td>
<td>67</td>
<td>58 51 43 36 35 59 ± 5 38 ± 3</td>
</tr>
<tr>
<td>Monophosphorylated (P1)</td>
<td>33 42 49 46 52 52</td>
<td>41 ± 5 50 ± 2</td>
</tr>
<tr>
<td>Diphosphorylated (P2)</td>
<td>0</td>
<td>0 12 12 13 0 12 ± 1</td>
</tr>
</tbody>
</table>

* Values are from three separate experiments.

**TABLE I** Distribution of unphosphorylated and phosphorylated forms of myosin light chains in unstimulated and stimulated RBL-2H3 cells.

The stoichiometry of phosphorylation of the RBL-2H3 myosin heavy chain was calculated by cutting out the heavy and light chains from the same one-dimensional SDS-polyacrylamide gel and determining the radioactivity of each by Cerenkov counting. Knowing that the radioactive phosphate in the myosin light chain was equivalent to a stoichiometry of 0.41 mol of phosphate/mol of light chain in unstimulated cells, we calculated the stoichiometry of phosphorylation of myosin heavy chain in these cells as 1.04 mol of phosphate/mol of heavy chain. Following 10 min of antigenic stimulation this increased to approximately 1.61 mol of phosphate/mol of heavy chain. Above we showed that the phosphate on the heavy chain in unstimulated cells was divided among three major tryptic phosphopeptides (Fig. 3). Following antigenic stimulation, these phosphopeptides did not change in intensity, although a single new tryptic phosphopeptide containing only phosphoserine appeared. The appearance of this phosphopeptide has been demonstrated to be due to protein kinase C activation. We calculated a stoichiometry of 0.57 mol of phosphate/mol of heavy chain due to protein kinase C activation assuming that the phosphopeptide contains a single serine (see Table II).

**TABLE II** Stoichiometry of myosin phosphorylation and histamine secretion from unstimulated and DNP-BSA-stimulated (10 min) RBL-2H3 cells.

<table>
<thead>
<tr>
<th>Cell batch Light chain sites</th>
<th>Heavy chain sites</th>
<th>Secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>0.41 0.37 0.37</td>
<td>0.41 0.37</td>
</tr>
<tr>
<td>Stimulated</td>
<td>0.37 0.38 0.38</td>
<td>0.37 0.38</td>
</tr>
</tbody>
</table>

* PKC, protein kinase C.

**TABLE II** Stoichiometry of myosin phosphorylation and histamine secretion from unstimulated and DNP-BSA-stimulated (10 min) RBL-2H3 cells.

2 S. Kawamoto, unpublished observation.
equivalent to 0.4 mol of phosphate/mol of MLCK site. We then measured the radioactive phosphate at the MLCK site and protein kinase C site for each time point following antigenic stimulation and used the specific activity derived above to determine the stoichiometry of phosphorylation for each site.

As shown in Fig. 6 the phosphate content at the MLCK site remained fairly constant during the 30-min time course. There was a small but reproducible rise in the first minute after antigenic stimulation that correlated with an increase in cell calcium. In contrast, the amount of phosphate in the protein kinase C site, initially 0, reached a maximum of 0.7–0.8 mol of phosphate/mol of light chain between 10 and 20 min. Fig. 6 also shows a time course for histamine secretion. Note that secretion was most rapid during the first 10 min of protein kinase C phosphorylation.

The data in Table I and the time course shown in Fig. 6 were derived from batches of RBL-2H3 cells that were harvested months apart. Table II compares these two batches with respect to histamine secretion and stoichiometry of myosin heavy chain and light chain phosphorylation following 10 min of stimulation by DNP-BSA. Of note is the finding that the extent of myosin light chain phosphorylation at the site phosphorylated by protein kinase C appears to correlate with the extent of histamine release in that 0.38 mol of phosphate/mol of protein kinase C site were incorporated into cells releasing 23% of their total histamine and 0.7 mol of phosphate/mol of protein kinase C site were incorporated into cells releasing 44% of their histamine. A similar increase in the stoichiometry of phosphorylation was also noted for the site phosphorylated by protein kinase C in the myosin heavy chain (0.57 mol of phosphate/mol of heavy chain for the cells secreting 23% and 1 mol of phosphate/mol of heavy chain in the case of those secreting 44%).

Both sets of experiments demonstrate the stoichiometry of phosphorylation of the sites on both the myosin heavy chains and light chains that were phosphorylated prior to stimulation did not appear to change following antigenic stimulation.

Myosin Light Chain Phosphorylation in Permeabilized Cells—Previous work has shown that after permeabilization with the streptococcal toxin, streptolysin O, RBL-2H3 cells still retained a substantial phosphoinositide and secretory response to DNP-BSA (22). Therefore, it was of interest to determine whether the same pattern of myosin phosphorylation that was found in intact cells would be evident in permeabilized cells, since these cells should permit a more direct assessment of the role of myosin in cell secretion. Fig. 7 shows time courses for the secretion of [3H]5-HT from RBL-2H3 cells that have been made permeable by streptolysin O as well as from stimulated intact cells. In these experiments [3H]5-HT release was monitored rather than histamine because of the ease of quantitating the former. Previous work has shown that the release of these two agents is equivalent in these cells (32).

The figure shows that DNP-BSA can initiate secretion from permeabilized cells that have been prelabeled with [32P] and that the time course of this release resembles that found with intact cells. The figure also shows that, unlike intact cells, the permeabilized cells release approximately 20% of their [3H]5-HT in 20 min in the absence of stimulation. The response of these cells to the nonhydrolyzable analog of GTP, GTPγS (45% release in 20 min), confirmed that these cells have been effectively permeabilized as GTPγS will only in-

---

**Fig. 6.** Time course of histamine release and stoichiometry of phosphorylation of the MLCK site and protein kinase C site in the myosin light chains. Phosphopeptides corresponding to the site phosphorylated by MLCK and the site phosphorylated by protein kinase C (PKC) were separated from tryptic peptide maps, assayed for [32P] radioactivity, and the stoichiometry calculated as described in the text. The time course of incorporation of radioactive phosphate catalyzed by each enzyme is depicted along with the percentage of histamine released.

---

**Fig. 7.** Time course of serotonin release from permeabilized and intact cells. The time course of [3H]5-HT release from intact (dashed line) and permeabilized (solid line) cells is shown. The extent of secretion was determined at the times indicated for control (▲) and stimulated (●) cells. The concentrations of DNP-BSA used for intact cells (20 ng/ml) and for permeabilized cells (100 ng/ml) were optimal for secretion.

---

**Fig. 8.** Autoradiograms of two-dimensional maps of tryptic digests of myosin light chains from permeabilized cells. The [32P]-labeled myosin light chains were cut from SDS-polyacrylamide gels and subjected to tryptic hydrolases and two-dimensional phosphopeptide mapping as described above. Cells were permeabilized in the presence of EGTA to buffer the calcium to virtually 0 PM (control) or were permeabilized in the presence of EGTA and calcium (to buffer free calcium to 1 μM) and 100 ng/ml DNP-BSA (stimulated). The numbers indicate the phosphopeptides that comigrated with the phosphopeptides obtained from platelet and turkey gizzard myosin light chains that were phosphorylated in vitro by MLCK (1) and protein kinase C (2). The unlabeled phosphopeptide to the left and slightly above the one labeled 2 is thought to contain the same phosphoserine residue phosphorylated by protein kinase C, but results from a different tryptic cleavage (34). X indicates the origin, E the direction of electrophoresis, and C the direction of chromatography.
Myosin Phosphorylation and Histamine Secretion

Duc secretion from permeabilized cells (22).

Fig. 8 shows autoradiograms of tryptic peptide maps of the myosin light chains from 32P-labeled cells before (control) and after 5 min of stimulation with DNP-BSA. The pattern, in general, is similar to the maps obtained after stimulation of intact cells in that in control cells the major phosphopeptide (1) is the one phosphorylated by MLCK. Following stimulation by DNP-BSA a second phosphopeptide (2) appears, which is known to be phosphorylated by protein kinase C.

**Discussion**

Previous work has shown that activation of rat basophilic leukemia (RBL-2H3) cells with DNP-BSA results in an increase in phosphatidylinositol turnover which leads to the generation of inositol phosphates and diacylglycerol (13). Thus, it is reasonable to postulate that RBL-2H3 cell stimulation would result in activation of protein kinase C, since diacylglycerol is known to activate this enzyme (35) and antigen-induced translocation of the enzyme has been demonstrated in these cells (14).

In this paper we document and quantitate the time course of RBL-2H3 cell myosin phosphorylation following aggregation of the receptors for IgE by DNP-BSA. Our purpose was to study changes in myosin phosphorylation that accompany stimulation. We were particularly interested in initiating RBL-2H3 cell secretion using a more physiologically relevant stimulus, for example one that causes receptor aggregation (12), rather than simply treating the cells with nonphysiological reagents such as phorbol esters in combination with a calcium ionophore.

Our results show that prior to stimulation with DNP-BSA there is already phosphate covalently bound to the myosin heavy chain and to the 20-kDa light chain. In the case of the 20-kDa myosin light chain, all of the phosphate is confined to a single tryptic peptide, which can be identified as the serine site phosphorylated by MLCK in both turkey gizzard smooth muscle and human platelet myosin (9, 10, 34). Following RBL-2H3 cell stimulation there is *de novo* phosphorylation of a serine site that has previously been shown to be phosphorylated by protein kinase C in turkey gizzard and human platelet myosin (34).

A similar scenario is observed for the myosin heavy chain. Unstimulated cells contain myosin heavy chains that yield a number of phosphorylated tryptic peptides which can also be observed following cell stimulation. However, following antigen stimulation, a single new tryptic phosphopeptide appears. Although we cannot positively identify the enzyme(s) responsible for catalyzing the phosphorylation of the heavy chain in unstimulated cells, there is evidence that it is not protein kinase C. Phosphorylation of platelet myosin by protein kinase C in vitro resulted in one major phosphopeptide (Fig. 3) which did not comigrate with any of the phosphopeptides generated by tryptic digestion of myosin heavy chains from unstimulated RBL-2H3 cells. It did comigrate, however, with the single new phosphopeptide that was generated from the myosin heavy chain of RBL-2H3 cells following antigenic stimulation (Fig. 3 and Ref. 34). Moreover, this same phosphopeptide was the only one generated from platelet myosin heavy chain following treatment of human platelets with phorbol esters (34). Thus, in this paper we demonstrate the receptor-mediated *in situ* phosphorylation of RBL-2H3 cell myosin heavy chain and light chain by protein kinase C.

In vitro studies using either smooth muscle myosin (9, 10) or human platelet myosin (34) show that protein kinase C can phosphorylate three sites in the 20-kDa myosin light chain, serine 1, serine 2, and threonine 9. In *situ* phosphorylation of both platelets and RBL-2H3 cells shows no evidence for threonine phosphorylation (Ref. 34 and this manuscript). Moreover, both of these studies also show evidence for myosin heavy chain phosphorylation following activation of protein kinase C.

Phosphorylation of the myosin heavy chains has been documented in vertebrate nonmuscle cells such as lymphocytes (36), leukemic myeloblasts (37), bovine brain cells (38, 39), macrophages (40), intestinal brush border cells (41) as well as myosin heavy chain from aorta smooth muscle cells (42). It has also been described in invertebrate cells such as *Acanthamoeba* (43), *Dictyostelium* (44), and molluscan catch muscle (45). It has been shown that the brain and chicken intestinal brush border myosin heavy chains can be phosphorylated by casein kinase II and calmodulin-dependent protein kinase II, respectively (38, 41). However, only in platelets has it been shown that there is a significant phosphorylation of the myosin heavy chain in intact cells following stimulation with phorbol esters, but even here the role of this phosphorylation in relation to platelet function remains obscure (34).

What, if any, is the role of myosin phosphorylation in the release of histamine from RBL-2H3 cells? There are three sites of phosphorylation to be considered: the sites on the myosin light chains phosphorylated by MLCK and protein kinase C and the site on the heavy chains phosphorylated by protein kinase C. The site phosphorylated by MLCK on the myosin light chains (peptide 1 in Fig. 2) is phosphorylated to 0.4 mol of phosphate/mol of light chain, and the net amount of phosphate does not change significantly during antigenic stimulation and cell secretion.

Phosphorylation of the MLCK site is usually associated with an increase in contractile activity in smooth muscle and nonmuscle cells (1-3). In the case of aorta smooth muscle myosin an important manifestation of phosphorylation is increased filament formation *in vitro* (46), which might be used to translocate granules in the cytoplasm. However, the relatively high level of phosphorylation at the MLCK site with no further change in net phosphorylation following stimulation in RBL-2H3 cells suggests that, if phosphorylation at this site is related to granule movement, it is not sufficient for granule release.

The most striking observation in these studies is that antigenic stimulation results in the incorporation of significant amounts of phosphate into sites on the myosin heavy chains and light chains known to be phosphorylated by protein kinase C. This finding is consistent with the translocation of protein kinase C in antigen-stimulated RBL-2H3 cells (14) and the phosphorylation of protein kinase C substrates in normal rat peritoneal mast cells (47). Moreover, the time course of myosin phosphorylation appears to correlate with the release of the granular contents from RBL-2H3 cells. The extent of protein kinase C phosphorylation of the myosin light chains and heavy chains also appears to correlate with the extent of secretion in that higher levels of myosin phosphorylation are found in cells that secrete a greater proportion of their granular contents.

The use of two-dimensional gel electrophoresis to separate the phosphorylated forms of the myosin light chains and subsequent peptide mapping of these forms has revealed that two monophosphorylated forms exist following antigenic stimulation: one phosphorylated by MLCK and another by protein kinase C. The monophosphorylated form catalyzed by protein kinase C could have arisen by phosphorylation of unphosphorylated myosin light chains by protein kinase C or by dephosphorylation of myosin light chains that had been previously phosphorylated by MLCK and protein kinase C (or by both pathways). In *situ*, the relative activities of the
various kinases and phosphatases will determine the proportion of each of these forms of the myosin light chains at any given time after stimulation. Results from in vitro studies with smooth muscle heavy meromyosin have shown little difference in the affinity of protein kinase C for myosin that was unphosphorylated or was previously phosphorylated by MLCK (8).

The available data are consistent with the view that histamine release is a two-step process requiring (a) phosphorylation of myosin by MLCK, which might be required for movement of granules and (b) phosphorylation of myosin light chains and heavy chains by protein kinase C, which may be required to dissociate myosin filaments, particularly at the cortical area so that granules can approach the cellular membrane. With respect to granule movement, in vitro studies with smooth muscle myosin have demonstrated two effects of the phosphorylation of the myosin light chain by protein kinase C: a marked decrease in the affinity of myosin previously phosphorylated by protein kinase C for MLCK and a decrease in the actin-activated MgATPase activity of myosin that had already been phosphorylated by MLCK (8, 10). Since previous work by several laboratories demonstrated that phosphorylation of the myosin light chains by MLCK enhanced filament formation (46, 48, 49), the subsequent activation of protein kinase C may be a mechanism to prevent further filament formation by rendering the myosin light chain a poorer substrate for MLCK and thus act to down-regulate secretion. It could also act to destabilize filaments by phosphorylating myosin that had previously been phosphorylated by MLCK. in vitro studies with smooth muscle heavy meromyosin support this effect (50). There are at present no in vitro studies available on the effects of vertebrate nonmuscle myosin heavy chain phosphorylation by protein kinase C. However, it is reasonable to speculate that protein kinase C phosphorylation of vertebrate nonmuscle myosin heavy chains, similar to phosphorylation of myosin heavy chains in protozoan systems (44, 45), might result in filament instability, thereby permitting the release of histamine and serotonin from RBL-2H3 cells.

The results with permeabilized cells are qualitatively similar to those obtained with intact cells with respect to secretion and phosphorylation of myosin light chains by MLCK and protein kinase C. Whether the apparent decrease in phosphorylation at the MLCK site following antigenic stimulation is due to differences in rates of loss of the two kinases from permeabilized cells, activation of phosphatases, dilution of radiolabeled ATP with unlabeled ATP in the media, or a combination of some of the above is uncertain (see Fig. 8). However, the important point to note is that antigen-stimulated myosin phosphorylation at the protein kinase C site was still demonstrable in permeabilized cells. The ability of specific inhibitory peptides to enter these permeabilized cells should allow us to address the individual role of each of these enzymes in the secretory process and thus determine if a causal relationship exists between myosin phosphorylation and secretion.

Acknowledgments—We are grateful to Henry Metzger for the supply of DNP-BSA and DNP-specific IgE. We also wish to acknowledge James R. Gillette, James R. Sellers, Sachio Kawamoto, and Mary Anne Conti for advice and helpful discussions and Catherine S. Magruder for tireless editorial assistance.

REFERENCES


