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Antigen-induced increase in protein kinase C activity in plasma membrane of mast cells

(IgE/quin-2/diacylglycerol/histamine release)

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ABSTRACT **Bridging of cell-bound IgE antibody molecules on colony-stimulating factor-dependent mouse mast cell line (PT-18) cells by multivalent antigen induces phospholipid methylation, a transient rise in intracellular cAMP, intracellular mobilization and uptake of Ca^{2+}, and formation of diacylglycerol followed by histamine release. Exposure of the sensitized cells to antigen also induces a substantial increase in protein kinase C activity in the plasma membrane, which is accompanied by a slight decrease in the enzyme in cytosol. Protein kinase C activity in the membrane fraction reached maximum within 30 sec after antigen challenge and then gradually declined. The increase of the enzyme activity in the membrane could not be explained by a shift of the enzyme from cytosol, and it suggested that bridging of IgE–receptor may induce a modulation of existing enzyme to a state of higher catalytic activity. Phorbol 12-myristate 13-acetate also induced a rapid but persistent increase in protein kinase C activity in the membrane fraction of mast cells. However, the increase in the enzyme activity in the membrane was accompanied by a marked decrease in the enzyme in cytosol.**

Mast cells bear receptors for IgE, and cross-linking of cell-bound IgE antibody molecules by multivalent antigen triggers the release of a variety of chemical mediators (1). Previous studies on the biochemical mechanisms of mast cell activation revealed that bridging of IgE receptors induces phospholipid methylation (2), a transient increase in cAMP (3), mobilization of intracellular Ca^{2+} (4) and Ca^{2+} influx (2, 5), and an enhancement of phosphatidylinositol turnover (6–8). Since diacylglycerol, a product of phosphatidylinositol metabolism, is a potent activator of Ca-activated phospholipid-dependent protein kinase (protein kinase C) (9), we speculated that bridging of IgE receptors may induce the activation of protein kinase C in mast cells. Kraft and Anderson (10, 11) reported that the activation of this enzyme in murine thymoma EL 4 cells and parietal yolk sac cells by phorbol 12-myristate 13-acetate (PMA) is accompanied by redistribution of the enzyme from cytosol to plasma membrane. The present experiments were therefore undertaken to determine whether the bridging of cell-bound IgE antibody molecules may induce the redistribution of protein kinase C in mast cells. Colony-stimulating factor-dependent mouse mast cell line PT-18 (12) was sensitized with mouse monoclonal IgE antibody specific for the 2,4-dinitrophenyl (DNP) group, and the sensitized cells were exposed to DNP derivatives of human serum albumin (HSA). The results show that cross-linking of cell-bound IgE antibody molecules induces a marked increase in protein kinase C activity in the plasma membrane.

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MATERIALS AND METHODS

Mast Cells, IgE Antibody, and Antigen. PT-18 cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, 50 μM 2-mercaptoethanol, and antibiotics. The culture medium was enriched with 3–5% (vol/vol) of medium conditioned by the culture supernatant of a cloned inducer T-cell line Ly-$1^{+}2^{-}$/9 (13), which contained mouse mast cell growth factor. The conditioned medium was kindly supplied by D. Rennick and F. Lee (DNAX Research Institute of Molecular and Cellular Biology). On average, PT-18 cells bear $2.57 \pm 0.30 \times 10^5$ IgE receptors per cell. PT-18 cells were passively sensitized with monoclonal IgE antibody specific for DNP, which was derived from hybridoma cell line H-1 DNP-ε-26 (14). Purified mouse IgE antibody employed in the present experiments is the same preparation as that described in a previous article (15). For passive sensitization, purified IgE was added to cultures of PT-18 cells at 10 μg/ml, and the cells were kept for 12–14 hr at 37°C. By this procedure, IgE receptors on PT-18 cells are saturated with mouse IgE antibody. The cells were washed twice and resuspended in Tyrode solution, pH 7.4, containing 124 mM NaCl, 4 mM KCl, 0.64 mM NaH_2PO_4, 1.6 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.1% gelatin, 5.5 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), and 5 mM 4-morpholineethanesulfonic acid (Mes). Phosphatidylserine (Supelco, Bellefonte, PA) was dispersed in the solution at 10 μg/ml. The sensitized cells were challenged with DNP-HSA, which contained 21 DNP groups per HSA molecule. Preliminary experiments showed that the concentration of DNP-HSA for maximal histamine release from the sensitized PT-18 cells was 0.01 μg/ml. Unless otherwise specified, this concentration of DNP-HSA was employed for antigen challenge of sensitized PT-18 cells.

Measurement of Phospholipid Methylation, Intracellular cAMP, ^{45}Ca Uptake, and Histamine Release. Activation of methyltransferases in PT-18 cells was detected by measuring incorporation of [^{3}H]methyl groups into phospholipids. Cells sensitized with IgE antibody were incubated in Tyrode solution containing 2 μM L-[*methyl*-^{3}H]methionine (15 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) and then challenged with DNP-HSA. Detailed procedures for the measurement of [^{3}H]methyl groups incorporated in the lipid fraction were described in a previous article (2).

Intracellular cAMP was determined by double antibody radioimmunoassay using 2′-*O*-succinyl-([^{125}I]iodotyrosine methyl ester) cAMP (New England Nuclear). Extraction of cAMP and details of the assay procedures were described in a previous article (3). ^{45}Ca influx into PT-18 cells was measured by the method of Foreman *et al.* (16), and hista-

Abbreviations: PMA, phorbol 12-myristate 13-acetate; DNP, 2,4-dinitrophenyl; HSA, human serum albumin.

mine release from the cells was measured by the automated technique of Siraganian (17).

Measurement of Intracellular Ca^{2+} by Quin-2 Fluorescence. Quin-2 fluorescence (18) was recorded by using a model 8000 photon-counting spectrofluorometer from SLM Instruments (Urbana, IL) with a temperature-controlled cuvette and a magnetically driven stirrer. PT-18 cells (5×10^8 cells) were loaded with quin-2 by incubating the cells with 100 μM quin-2 acetoxymethyl ester for 10 min. The cell suspension was diluted 1:10 with complete Tyrode solution, kept 45 min, and then washed twice with the solution. A cell suspension (5×10^6 cells per ml) was placed in the cuvette described above, and agents were added by microsyringe directly into the cuvette, without interrupting recording. Fluorescence excitation and emission wavelengths were 339 nm and 492 nm, respectively. The concentration of intracellular Ca^{2+} was calculated by the method described by Tsien *et al.* (18).

Determination of Diacylglycerol. [^{3}H]Glycerol (1 Ci/mmol; Amersham) was added to a suspension of PT-18 cells (50 μCi per 10^6 cells) during passive sensitization with IgE antibody. After washing, the cells were challenged with DNP-HSA. At appropriate intervals, the reaction was stopped by adding 0.01 M EDTA and the cell suspension was immediately centrifuged. Lipids were extracted from cell pellets by the method of Bligh and Dyer (19). Samples dried under nitrogen were dissolved in 20 μl of chloroform containing 1,2-dipalmitoyl-*rac*-glycerol (1 mg/ml) and were analyzed by thin-layer chromatography on silica G plates (Analtech, Newark, DE), using dichloroethylene/ethanol (98/2, vol/vol) (20). The radioactivity of the diacylglycerol spot was measured in a scintillation spectrometer.

Determination of Protein Kinase C Activity. The activity of the enzyme was measured by its ability to transfer ^{32}P from [γ-^{32}P]ATP into histone H1 (9). The assay mixture is a 40 mM Tris·HCl buffer, pH 7.5, containing histone H1 at 40 μg/ml, 50 μM ATP (including [γ-^{32}P]ATP), 10 mM 2-mercaptoethanol, 0.4 mM EGTA, 10 mM $MgCl_2$, 2 mM $CaCl_2$, phosphatidylserine at 40 μg/ml, and 1,2-dioleoylglycerol at 2 μg/ml; 50 μl of a sample to be assayed was added to the mixture in a total volume of 250 μl. To assess Ca^{2+} and phospholipid-dependent enzyme activity, assays were run without Ca^{2+} and/or phosphatidylserine, and protein kinase C activity was calculated as the difference. The mixtures were incubated for 10 min at 30°C, and the reaction was stopped by the addition of 2 ml of ice-cold 25% trichloroacetic acid. Precipitates were collected on Whatman microfiber filters (GF/F) and washed three times with 2 ml of 25% trichloroacetic acid. Radioactivity of each filter was determined in a scintillation spectrometer using Dimiscint (National Diagnostics, Somerville, NJ) as scintillant. Since the phosphorylation of histone was linear over 10 min, the activity of protein kinase C was expressed as pmol of ^{32}P incorporated into histone H1 per min per 10^7 cells.

Preparation of Subcellular Fractions. To determine protein kinase C activity in cytosol and membrane fractions, subcellular fractions of mast cells were prepared as previously described (11). About 1–2 $\times 10^7$ PT-18 cells were suspended in 1 ml of extraction buffer (50 mM Tris·HCl buffer, pH 7.5, containing 50 mM 2-mercaptoethanol, 10 mM phenylmethylsulfonyl fluoride, and 2 mM EGTA) and disrupted at 4°C by sonication for 10 sec and 5 sec in a Heat System/Ultrasonics water bath sonicator (Plainview, NY) at output of 50 V. Disrupted cells were centrifuged at 100,000 $\times$ *g* for 1 hr and supernatant was used as a cytosol fraction. The pellet (particulate fraction) was resuspended in 1 ml of extraction buffer and sonicated again to obtain a homogeneous suspension. The suspension was stirred with 1% Triton X-100 at 4°C for 90 min, and applied to a DEAE-cellulose column (Whatman DE-52; 0.5 $\times$ 2 cm). After washing with 10 ml of 5 mM Tris·HCl buffer, pH 7.5, containing 1 mM EGTA and 50 mM 2-mercaptoethanol, the column was eluted with 1 ml of the same Tris buffer containing 0.15 M NaCl. The eluate was employed to determine protein kinase C activity in the particulate fraction.

To obtain membrane fractions, 1×10^8 PT-18 cells were sonicated in 1 ml of extraction buffer. The suspension was centrifuged at 1200 $\times$ *g* for 5 min to remove nuclei. The supernatant was layered onto a discontinuous sucrose gradient consisting of 30%, 40%, and 50% sucrose, which contained 1 mM EGTA and 50 mM 2-mercaptoethanol at pH 7.5. After centrifugation in a Beckman SW 50 rotor at 120,000 $\times$ *g* for 1 hr, bands formed at each of the sucrose interfaces were recovered (21, 22). Analysis of marker enzymes such as 5′-nucleotidase and monoamine oxidase revealed that only the top two bands contained plasma membranes.

Reagents. Histone H1 (type III-S), ATP, 1,2-dioleoylglycerol, 1,2-dipalmitoyl-*rac*-glycerol, Tris, Hepes, 2-mercaptoethanol, dimethyl sulfoxide, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride, and PMA were purchased from Sigma. Triton X-100 was from Baker. Quin-2 acetoxymethyl ester was purchased from Lancaster Synthesis (Lancashire, England).

RESULTS

Initial Biochemical Events Induced by Antigen Stimulation. DNP-HSA was added to sensitized PT-18 cells, and kinetics of phospholipid methylation, changes in cAMP levels, ^{45}Ca uptake, and histamine release were determined. As shown in Fig. 1, both the incorporation of [^{3}H]methyl group into phospholipids and initial rise in cAMP reached a maximum 30 sec after antigen challenge, and both values declined to baseline levels by 2 min. ^{45}Ca uptake reached a maximum at 2 min and histamine release reached a maximum at 5 min. Antigen challenge of the sensitized cells caused a rapid increase in intracellular Ca^{2+}. As shown in Fig. 2, quin-2 fluorescence prior to antigen challenge represents 40–70 nM free calcium. After antigen stimulation, intracellular Ca^{2+} rose to 160 to 200 nM within 2 min, and this level was maintained by 5 min. It should be noted in Fig. 2 that the increase in quin-2 signal is composed of two steps; an initial rise occurred within 10–15 sec, and this was followed by a second, sustained, rise from 30 sec to 2 min. The addition of 3 mM EGTA to the sensitized cells 1 min prior to antigen challenge did not affect the initial rise but completely suppressed the second, sustained, increase in quin-2 fluorescence. This finding, together with parallelism in kinetics of ^{45}Ca uptake and the second increase in quin-2 signal (cf. Fig. 2), indicate that the initial rise is due to mobilization of intracellular Ca^{2+}, while the second rise is due to influx of extracellular Ca^{2+}.

We have also determined whether antigen challenge of the sensitized cells induced the formation of diacylglycerol. As shown in Fig. 2, substantial amount of diacylglycerol was detected within 30 sec after antigen challenge. The kinetics of diacylglycerol formation paralleled those of histamine release. It was noted that diacylglycerol in mast cells continued to increase over 10 min.

Activation of Protein Kinase C. In view of previous reports indicating that the activation of protein kinase C is accompanied by redistribution of the enzyme from cytosol to plasma membrane (10, 11), we determined protein kinase C activity in particulate and cytosol fractions at various intervals after antigen challenge. As shown in Fig. 3, protein kinase C activity in the particulate fraction increased 3- to 4-fold within 30 sec after antigen challenge and then gradually declined. The enzyme activity in the particulate fraction returned to the initial level by 10 min. In contrast, protein kinase C activity in cytosol concomitantly diminished over the same period and recovered to the original levels by 10

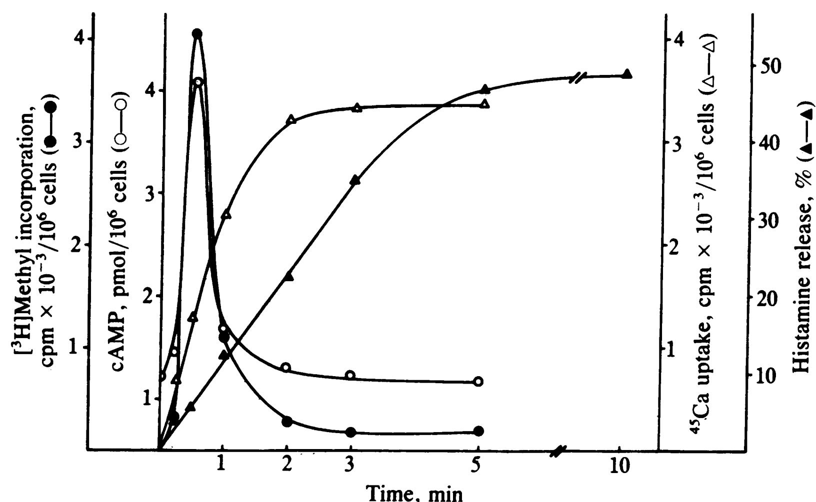

FIG. 1. Kinetics of [^{3}H]methyl incorporation (●), cAMP rise (○), ^{45}Ca uptake (△), and histamine release (▲) induced by DNP-HSA. PT-18 cells were sensitized with mouse anti-DNP IgE antibody at 10 μg/ml, washed, and then challenged with DNP-HSA (0.01 μg/ml). The same cell preparation was used for all measurements. Each point is an average of duplicate measurements. [^{3}H]Methyl incorporation and ^{45}Ca uptake by unstimulated cells were 1428 cpm per 10^6 cells and 1110 cpm per 10^6 cells, respectively. Spontaneous histamine release from the cells was 8%. These values were subtracted from the experimental values. Two more experiments of the same design gave similar results.

min. However, the increase in protein kinase C activity in the particulate fraction cannot be explained by a shift of the enzyme from the cytosol, because a small decrease of cytosol-associated protein kinase C does not account for a greater increase in the particulate fraction. To confirm this finding, sensitized PT-18 cells were challenged with DNP-HSA and total protein kinase C activity in the homogenate of the cells was determined without fractionation. Protein kinase C activity increased 2.7-fold within 30 sec after the antigen challenge.

Experiments were carried out to confirm that protein kinase C activity in the particulate fraction is associated with plasma membranes. One-half of a suspension of sensitized cells was challenged with DNP-HSA, and the cells were sonicated 30 sec after the challenge. Particulate fractions from both challenged cells and control cells were then fractionated by centrifugation over a discontinuous sucrose gradient. In a preliminary experiment in which mast cells sensitized with ^{125}I-labeled IgE were fractionated, only the top two bands contained radioactivity and protein kinase C activity, together with membrane markers such as 5′-nucleotidase. It was found that the two fractions obtained from the antigen-stimulated cells contained 5-fold more protein kinase C activity than the same fractions of control cells. The results indicate that an increase in kinase activity occurred in plasma membrane after antigen challenge.

We have also determined whether the activation of protein kinase C depends on the concentration of antigen for stimulation. Aliquots of sensitized cells were challenged with various concentrations of DNP-HSA. Cells were disrupted 30 sec after the antigen challenge, and protein kinase C activity in the particulate fraction and cytosol was determined. The same sensitized cells were incubated with DNP-HSA for 5 min to measure histamine release. The results shown in Fig. 4 indicate that the concentration of DNP-HSA for maximal histamine release—i.e., 0.01 μg/ml—is optimal for maximal increase in protein kinase C activity in the particulate fraction, which is accompanied by concomitant decrease of the enzyme activity in the cytosol. It should be noted that both histamine release and the increase in kinase activity in the particulate fraction became less when sensitized cells were stimulated with DNP-HSA at 0.1 μg/ml.

The activation of protein kinase C by PMA in lymphocytes is accompanied by a shift of the enzyme from cytosol to plasma membrane (11). To confirm this observation with PT-18 cells, the sensitized mast cells were incubated with 50 ng of PMA, which is optimal for histamine release, and protein kinase C activity in the particulate fraction and cytosol was determined at indicated intervals (Fig. 5). The results clearly showed a rapid increase in protein kinase C activity in the particulate fraction within 30 sec, and this change was accompanied by a marked decrease in the enzyme activity in cytosol. It was also noted that these changes persist for 40 min, the period for which the observations were made. Exposure of the cells to dimethyl sulfoxide, which was used to dissolve PMA, failed to change protein kinase C activity (cf. Fig. 5). Results of repeated experiments on PMA and antigen stimulation are summarized in Table 1. The ratio of membrane-associated protein kinase C to that of cytosol increased 20-fold after stimulation with PMA, while the increase in the ratio was about 5-fold when the cells were stimulated by antigen. It should be noted,

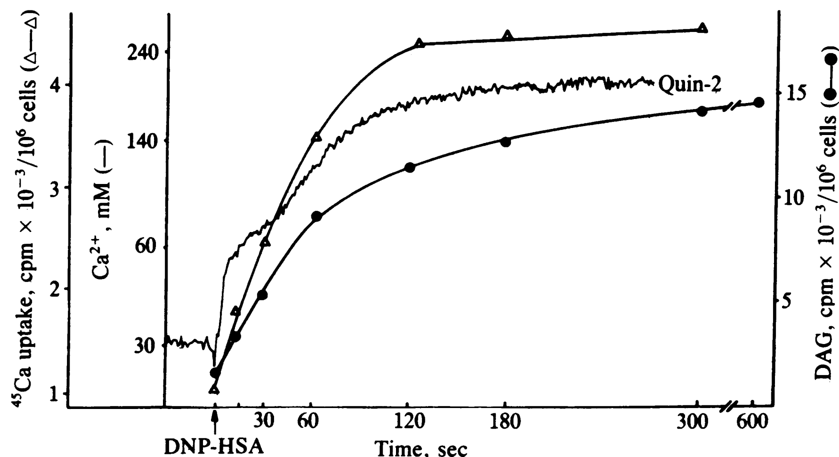

FIG. 2. Kinetics of ^{45}Ca uptake, diacylglycerol formation, and increase in cell-associated quin-2 fluorescence induced by DNP-HSA. PT-18 cells were sensitized with anti-DNP IgE antibody at 10 μg/ml, overnight. To determine the kinetics of diacylglycerol (DAG) formation, the cells were labeled with [^{3}H]glycerol. Aliquots of the cell suspension were challenged with DNP-HSA, and diacylglycerol generated in the cells at the indicated time was determined by thin-layer chromatography on silica G plates (●). The same sensitized cells were employed to measure ^{45}Ca uptake (△). An aliquot of sensitized cells was loaded with quin-2 and challenged with DNP-HSA, and changes in quin-2 signal were recorded.

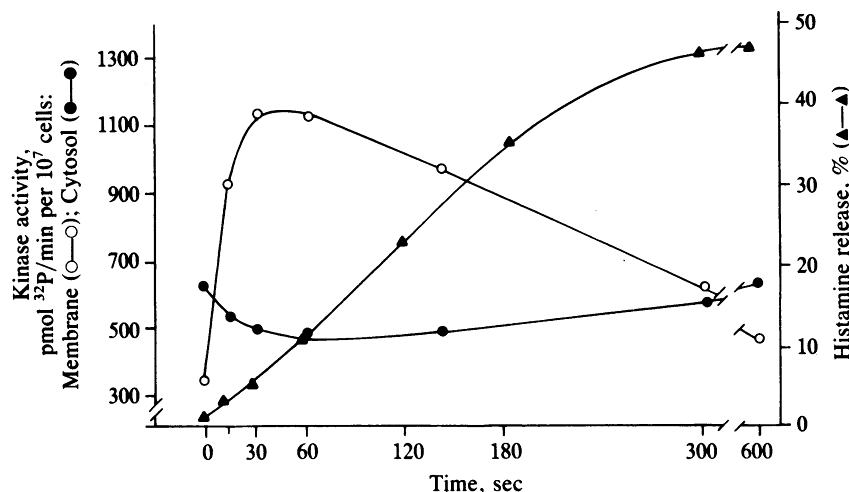

FIG. 3. Kinetics of protein kinase C activation. Sensitized cells were stimulated with DNP-HSA (0.01 μg/ml). The reaction was allowed to proceed for the indicated time before the reaction was terminated by the addition of ice-cold buffer (2.5 ml). The activity of protein kinase C in the particulate (○) and the cytosolic (●) compartments was determined. Data shown in the figure are the average of three different experiments. In a different set of sensitized cells, the rate of histamine release was determined.

however, that maximal levels of protein kinase C in the membrane fraction were comparable with antigen and PMA stimulation.

DISCUSSION

The present experiments clearly show that bridging of cell-bound IgE antibody molecules on PT-18 cells by multivalent antigen induces phospholipid methylation, cAMP rise, Ca^{2+} uptake followed by the formation of diacylglycerol, and histamine release. Kinetics of these biochemical events observed in PT-18 cells were similar to those observed in normal rat peritoneal mast cells (2). In both normal rat mast cells (24) and PT-18 cells, diacylglycerol content continued to increase for 10 min. The bridging of IgE-antibody molecules also induced mobilization of intracellular Ca^{2+}, as determined by quin-2 fluorescence. The results obtained in PT-18 cells were similar to those observed in rat mast cells sensitized with the same mouse IgE antibody (4). Thus, it appears that biochemical processes induced by bridging of cell-bound IgE antibody molecules on PT-18 cells represent those in normal mast cells.

Among the important findings obtained in the present experiments is that bridging of cell-bound IgE antibody molecules on PT-18 cells induces a substantial increase in protein kinase C activity in the plasma membrane, which is accompanied by a slight decrease in the enzyme in cytosol. It should be noted that the increase of the enzyme activity in the particulate fraction was much more than the decrease of the enzyme in the cytosolic fraction and that the total activity in the cells increased more than 2-fold after antigen stimulation. Thus, an increase in protein kinase C activity in the plasma membrane cannot be explained by a shift of this enzyme from cytosol to plasma membrane. On the other hand, redistribution of the enzyme from cytosol to plasma membrane was observed when PT-18 cells were stimulated by PMA. However, as the total activity of protein kinase C in the cell homogenate increased by 50% after stimulation with PMA, it is doubtful whether the shift from cytosol may totally explain the increase in the enzyme activity in plasma membrane.

It is believed that the activation of protein kinase C is accompanied by a shift of the enzyme from cytosol to plasma membranes (25). Since the bridging of IgE receptors on mast cells results in the formation of diacylglycerol and an increase in intracellular Ca^{2+}, it is reasonable to speculate that protein kinase C is activated as the result of receptor bridging. However, an increase in kinase activity observed in the present experiments cannot be explained by diacylglycerol formation, because the enzyme activity was measured in the presence of an optimal concentration of Ca^{2+}, diacylglycerol, and phosphatidylserine. Recently, Hamilton et al. (23) reported that γ interferon induces an enhancement of protein kinase C activity by as much as 5-fold in macrophages. In this system, changes in activity do not result from *de novo* synthesis of new enzymes but rather from a modulation of existing enzyme to a state of higher catalytic activity. We suspect that bridging of IgE–receptors on mast cells may induce similar modulation of the enzyme.

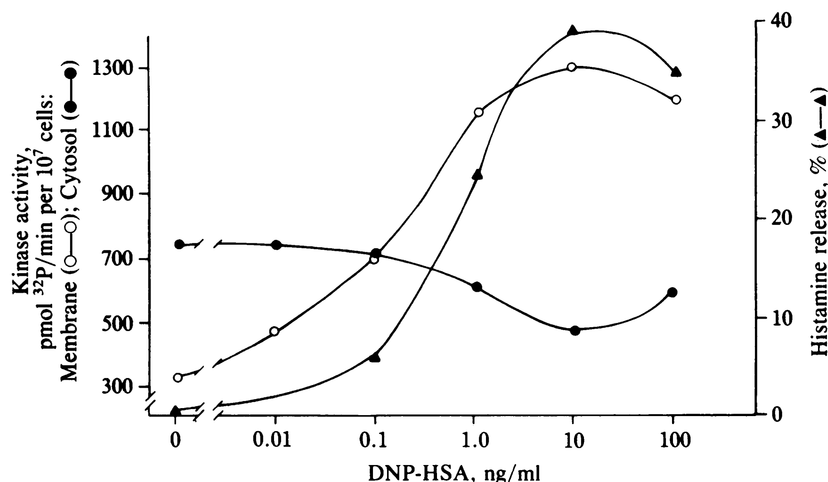

FIG. 4. Determination of an optimal concentration of antigen for the activation of protein kinase C. Sensitized PT-18 cells were incubated for 45 sec with various concentrations of antigen before the reaction was terminated by the addition of 2.5 ml of ice-cold buffer. The activity of protein kinase C associated with the particulate (○) and cytosolic (●) fractions was determined. Histamine release (▲) was determined on a separate set of cells. Spontaneous histamine release (5%) was subtracted from all values.

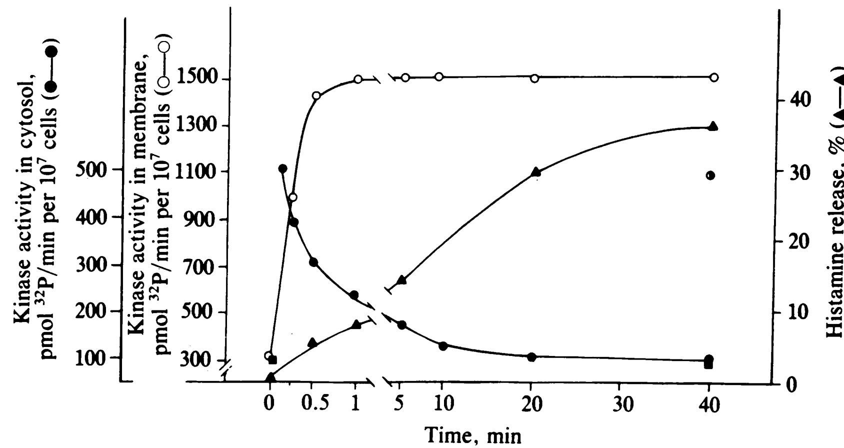

FIG. 5. Time course of protein kinase C activation with PMA. Cells were stimulated with PMA for the indicated time before the reaction was terminated. The amount of protein kinase C activity in the particulate (○) and the cytosolic (●) fractions was then determined. The addition of dimethyl sulfoxide (0.1%) to the cells failed to alter the enzyme activity in either the membrane (■) or the cytosolic (◑) fraction. Histamine release was determined on a separate set of cells. Spontaneous release did not exceed 5% and was subtracted from all values. The results are the average of data obtained in three experiments.

It is not known whether the modulation of protein kinase C is a consequence of the activation of the enzyme. Nevertheless, our recent experiments provided separate evidence that protein kinase C in PT-18 cells is activated when cell-bound IgE antibody molecules are bridged. In this experiment, sensitized PT-18 cells were labeled with ^{32}P and then stimulated with DNP-HSA. Analysis of cell lysate obtained either prior to or 30 sec after antigen challenge by two-dimensional sodium dodecyl sulfate gel electrophoresis (26) revealed that antigen stimulation enhanced phosphorylation of several proteins. Among these proteins, protein kinase C appears to be involved in the phosphorylation of at least two proteins, 50 and 52 kilodaltons, with pKa values of 4.8 and 4.9, respectively. The same proteins were phosphorylated when a cytosol fraction of cell lysate was incubated with [γ-^{32}P]ATP in the presence of phosphatidylserine and Ca^{2+} but not in the absence of either phosphatidylserine or Ca^{2+}. Further studies are required to identify these proteins.

A question remains whether the activation and/or modulation of protein kinase C is involved in the process of mediator release. The present experiments showed that an increase in protein kinase C activity in the plasma membrane reached maximum within 30 sec after antigen challenge, much earlier than the initiation of histamine release. It was also noted that an optimal concentration of antigen for histamine release induced maximal increase in kinase in the plasma membrane. A 10-fold increase in challenging dose resulted in a significant decrease both in histamine release and in the increase of protein kinase C activity. These results suggest that the modulation and activation of the enzyme may be involved in the process of mediator release; however, further studies are required before any conclusion can be drawn.

Table 1. Distribution of protein kinase C activity between cytosolic and particulate fractions

	Protein kinase C activity			
	^{32}P incorporation, pmol/min per 10^7 cells			
Stimulation*	Cytosol	Membrane	Total	Membrane/cytosol
None	564 ± 104	340 ± 44	904	0.6
PMA	114 ± 35	1442 ± 152	1556	12.6
DNP-HSA	431 ± 75	1375 ± 125	1806	3.2

Results are mean (±SD) of six experiments.
*PT-18 cells sensitized with mouse IgE antibody were incubated for 5 min with PMA at 0.1 μg/ml or for 30 sec with DNP-HSA at 0.01 μg/ml.

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