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INHIBITORY ACTION OF PHORBOL MYRISTATE ACETATE ON HISTAMINE SECRETION AND POLYPHOSPHOINOSITIDE TURNOVER INDUCED BY COMPOUND 48/80 IN MAST CELLS

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SUMMARY: Rat peritoneal mast cells which had been preincubated with phorbol myristate acetate (PMA, 10 - 100 ng/ml) for 5 min did not elicit the full histamine secretion induced by a potent secretagogue, compound 48/80. Furthermore, this PMA-treatment was found to inhibit the agonist-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in [^{32}P]labeled cells. However, it was also observed that the level of [^{32}P]PIP_2 was markedly reduced by 5 min-incubation with PMA. This suggests the enhanced hydrolysis of PIP_2 by PMA which was reflected in a greater formation of inositol trisphosphate (IP_3). These observations indicate that the formation of IP_3 may not be profoundly related to secretory response in mast cells. © 1985 Academic Press, Inc.

Substantial evidences have established a generally accepted theory that a tumor promoter, phorbol myristate acetate (PMA), is a direct activator of protein kinase C, which mediates cellular responses, as reviewed by Nishizuka (1). Recently, it has been suggested that PMA also may exert an inhibitory action on secretion and Ca^{2+} mobilization, dependent on its concentration. However, such inhibitory influence varies among different cell types. Histamine secretion is repressed by the high concentration of PMA in rat peritoneal mast cells (2) and basophilic leukemia cells (3), whereas there is no indication of inhibition of secretion in human platelets and HL60 cells under the same conditions (3). Furthermore, it is observed that preincubation of cells with PMA effectively inhibits the agonist-evoked Ca^{2+} mobilization, secretion and phosphoinositide

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Abbreviations: PIP_2; phosphatidylinositol 4,5-bisphosphate, IP_3; inositol trisphosphate, PMA; phorbol 12-myristate 13-acetate, PIP; phosphatidylinositol 4-phosphate, IP_2; inositol bisphosphate, PI; phosphatidylinositol, IP; inositol monophosphate, PA; phosphatidic acid, PC; phosphatidylcholine, PE; phosphatidylethanolamine, MCM; mast cell medium, Hepes; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, EDTA; ethylenediaminetetraacetic acid, BSA; bovine serum albumin, PAF; platelet-activating-factor, DMSO; dimethylsulfoxide.

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turnover. Naccache et al.(4) have shown that in rabbit neutrophils, PMA added prior to stimulation with a chemotactic peptide causes a significant inhibition of the subsequent increases in the lysosomal enzyme release and in the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$. MacIntyre et al.(5) report that the PMA-treatment for 2 min of human platelets inhibits thrombin-, PAF- and vasopressin-induced enhancement of $[Ca^{2+}]_i$ and phosphatidate production. In addition, suppressive effect of PMA in PC-12 cells has been demonstrated by Vicentini et al. (6), who obtained the finding that the cholinergic agonist, carbachol, is unable to induce normal responses of $[Ca^{2+}]_i$ rise and of overall phosphoinositide hydrolysis in the PMA-preincubated cells. Although the underlying mechanism(s) of such antagonistic properties of PMA is unknown, a plausible explanation was given in terms of the altered action of Ca^{2+} mobilizing machinery. Therefore, to get more insight into the mechanism(s), we have investigated the effects of PMA-pretreatment on histamine secretion and phosphoinositide turnover known to be implicated in the $[Ca^{2+}]_i$ control in rat peritoneal mast cells induced by compound 48/80.

MATERIALS AND METHODS

Isolation of rat mast cells: Mast cells were obtained from peritoneal cavity of Wistar rats and purified through BSA gradient centrifugation as previously described in detail (9), except that Hepes-buffered medium was used instead of phosphate-buffered medium. The viability of the cells was more than 90% as assessed by toluidine blue staining. All the wares used were of polyethylene or siliconized glass.

Histamine release: Purified mast cells (1-2 x 10^5 cells) were challenged with secretagogues for the designated periods of time, and histamine release was terminated by adding two volumes of 10 mM EDTA-containing mast cell medium (MCM) to the cell suspension. The contents of histamine in the supernatant and the pellet were determined by the method of Shore et al.(10) using Hitachi Fluorescence Spectrophotometer MPF-3. The percent release was expressed as the ratio of histamine content in the supernatant to the sum of supernatant and pellet.

Polyphosphoinositide metabolism: Purified mast cells were prelabeled with [^{32}P]orthophosphate (0.5 mCi/10^7 cells) for 60 min at 37°C, and washed with MCM. Radiolabeled mast cells (3-5 x 10^5 cells/0.5 ml) resuspended in MCM containig 1.0 mM $CaCl_2$ and 0.5 mM $MgCl_2$ were first incubated with PMA (10 or 100 ng/ml) for 5 min and then exposed to compound 48/80 (0.5 µg/ml) for the indicated periods of time at 37°C. Reaction was terminated by the addition of 2 ml of chloroform / methanol / conc. HCl (20/40/1, v/v) (11). and the lipids were extracted by the method of Bligh and Dyer (12). The polyphosphoinositides were separated on HPTLC impregnated with 1% potassium oxalate using the system of chloroform / acetone / methanol / acetic acid / water (40/15/13/12/8, v/v) (11). The areas corresponding to individual lipids located by autoradiography performed on X-Omat K film, were scraped into vials and the radioactivity was determined in liquid scintillation counter (Beckman LS-7500) (13).

Inositol phosphates analysis: Mast cells were labeled with [^{3}H]inositol (37.5 μCi/10^6 cells/0.1 ml) in Hepes-buffered MCM for 2 hr at 37°C under 5 % CO_2 / air (12). After radiolabeling, the cells were further incubated in the LiCl (10 mM to inhibit inositol-1-phosphatase)-containing medium for 30 min at 37°C, then challenged wih 100 ng/ml of PMA and the reaction was terminated by adding 3.76 volumes of chloroform / methanol / conc. HCl (20/40/1, v/v) (15). The aqueous phase was neutralized and diluted with water, an aliquot of which was employed for total radioactivity counting and the rest was applied onto AG-1x8, 100-200 mesh, formate form (Bio-Rad) column (0.6 x 4.0 cm) to elute free inositol. Glycerophosphoinositol, inositol phosphate (IP), inositol bisphosphate (IP_2), and inositol trisphosphate (IP_3) were sequentially eluted with 12 ml of 5 mM sodium tetraborate/60 mM ammonium formate, 16 ml of 0.2 M ammonium formate/0.1M formic acid, 6 x 4 ml of 0.4 M ammonium formate/0.1 M formic acid, and 8 x 2 ml of 1.0 M ammonium formate/0.1 M formic acid, respectively. Two milliliters of IP_3 fraction were first diluted with water to 4 ml, and the radioactivity in 4 ml of each fraction was counted in the gel phase using 60 % (v/v) Aquasol-2 (New England Nuclear) (16).

Materials: [^{32}P]orthophosphate was from New England Nuclear and [^{3}H]inositol was from Amersham. Phorbol 12-myristate 13-acetate (PMA), compound 48/80 and BSA (fraction V, essentially fatty acid free) were purchased from Sigma. HPTLC plates (5631) were products of Merck. A protein kinase C inhibitor, H-7 was kindly supplied by Dr. H. Hidaka, Mie University School of Medicine. X-Omat K film is a product of Kodak. All other chemicals were of reagent grade.

RESULTS AND DISCUSSION

When rat peritoneal mast cells were exposed to stimuli, either receptor-mediated (antigen), membrane-perturbing (compound 48/80) or by-passing (A23187, PMA), the active secretion has been known to be induced (16, 17), though the extent of secretion varies with their concentrations. However, unexpectedly the addition of PMA to the mast cell suspension prior to the stimulation with compound 48/80 was found to prevent the full secretion (Fig. 1). After mast cells were preincubated in the presence of PMA (10 and 100 ng/ml) for 5 min at 37°C, histamine secretion induced by compound 48/80 (0.5 μg/ml) was repressed to 78% and 65% of the maximal response observed without PMA-pretreatment, respectively. In addition, in the cells treated with H-7, an inhibitor of protein kinase C (17), there were no significant changes in the secretory activity. But addition of PMA to cells previously exposed to H-7 gave an indication of an enhanced histamine release (data not shown). This seems to be consistent with the finding by Inagaki et al.(17), who have demonstrated that human platelets exposed to H-7 displayed a greater serotonin release compared to untreated control platelets. Recent studies using PMA of several investigators have provided evidence which suggests that protein kinase C may serve as a dual regulator of cellular

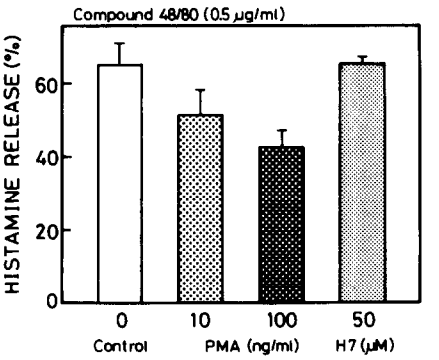

Fig. 1: The effect of preincubation with phorbol myristate acetate or H-7 on agonist-induced histamine secretion in mast cells. Rat peritoneal mast cells were preincubated either in the presence or absence of PMA (10 or 100 ng/ml) or H-7 (50 μM) for 5 min at 37°C. After 5 min-preincubation, the cells were challenged with 0.5 μg/ml of compound 48/80 for 10 sec, and the reaction was terminated by adding two volumes of 10 mM EDTA-containing MCM. Results are expressed as the percentage of released histamine to the total cell-associated histamine. Each value is the mean of triplicate determinations from two separate experiments. Bars indicate ± SD of three determinations or the range of two determinations.

responses (3-5). And the interpretation common to the inhibitory action by PMA on agonist-induced secretion is based on the repressed rise of cytosolic free Ca^{2+} concentration. Although the exact underlying mechanism(s) remains to be clarified, some speculations were made for this altered Ca^{2+} mobilization; closure of Ca^{2+} gate, activation of Ca^{2+} pump (3, 5) and inhibition of phosphoinositide hydrolysis (6).

Therefore, as an initial approach we have attempted to investigate whether PMA does affect phosphoinositide hydrolysis, since inositol trisphosphate (IP_3), a degradation product of phosphatidylinositol 4,5-bisphosphate (PIP_2) is known as a novel second messenger for Ca^{2+} release from non-mitochondrial organelles, probably endoplasmic reticulum (18). As shown in Fig. 2, upon stimulation with compound 48/80 alone, [^{32}P]radioactivity in PIP_2 elicited a transient decrease at 10 sec after the addition of the stimulant. The level of [^{32}P]labeled phosphatidylinositol 4-phosphate (PIP) showed an increase followed by the plateau. There was no changes in PI, whereas phosphatidic acid (PA) increased progressively to a small but significant extent. On the other hand, in the PMA-pretreated mast cells, we were unable to observe any indication of degradation of [^{32}P]labeled PIP_2. It is to be noted that the initial level at zero time of

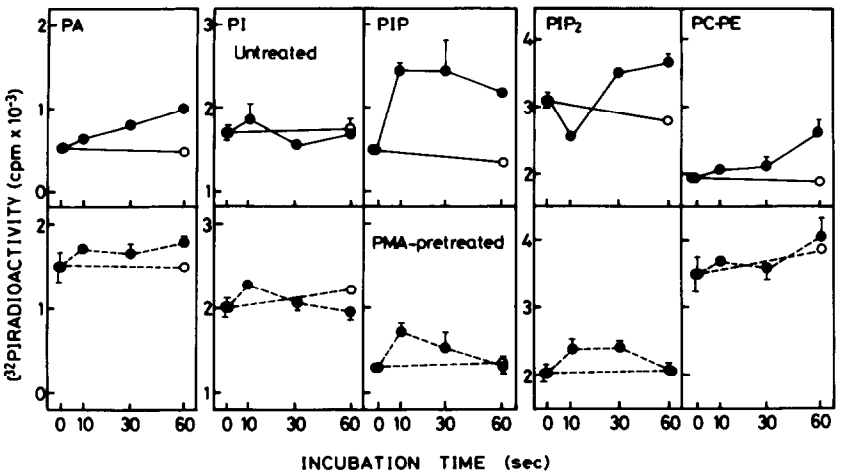

Fig. 2: Effect of PMA-preincubation on polyphosphoinositide metabolism in [^{32}P]orthophosphate-labeled mast cells stimulated with compound 48/80. Rat peritoneal mast cells, prelabeled with [^{32}P]orthophosphate, were preincubated in the presence (lower panel) or absence (upper panel) of PMA (100 ng/ml) for 5 min at 37°C and then exposed to 0.5 μg/ml of compound 48/80 for the indicated periods of time. Results are expressed as the mean of duplicate determinations from a representative of two experiments showing similar data. Ranges refer to duplicate analysis.

[^{32}P]radioactivity in PIP_2 was considerably lower than that in the mast cells stimulated with compound 48/80 alone (without PMA-pretreatment). There were great increases in PA and phosphatidylcholine (PC)/phosphatidylethanolamine (PE). Thus, the results of [^{32}P] labeling experiments indicated the PMA-mediated prevention of PIP_2 breakdown.

As described above, we have obtained the unexpected finding that the preincubation with PMA for 5 min causes marked decrease in the initial level before stimulation of [^{32}P]PIP_2 and profound increases in PA and PC/PE. Consequently, more detailed analyses of changes in [^{32}P] distribution in each phosphoinositide were performed for mast cells treated with PMA (100 ng/ml). Data are presented in Fig. 3, showing that within 30 sec the levels of both PIP_2 and PIP considerably increase and then decrease. Such increases of polyphosphoinositides were also observed with human platelets (21, 22) and human neutrophils (23), suggesting the enhanced phosphorylation of PI and PIP via PI kinase and PIP kinase, respectively. Following the rapid increase of these polyphosphoinositides, they began to be degraded and were reduced to much lower levels than their original levels. In contrast, PA and PC/PE exhibited the drastic increase in [^{32}P]radio-

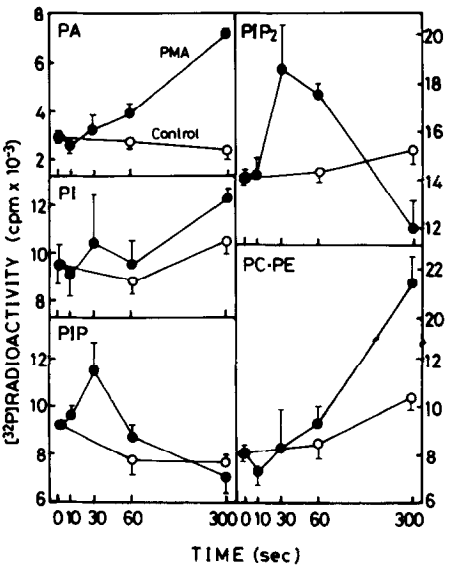

Fig. 3: Polyphosphoinositide metabolism in [^{32}P]prelabeled-mast cells treated with PMA. Rat peritoneal mast cells, prelabeled with [^{32}P]orthophosphate, were incubated with PMA (100 ng/ml) or DMSO (0.03%) at 37°C for indicated times. Each value is the mean of duplicate determinations from a single experiment performed in duplicate. Bars indicate ± SD of quadriplicate determinations.

activity, which reflected the augmented de novo synthesis, as reported with other cells (24-26). Since the great decline of PIP_2, as assessed by both [^{32}P]phosphate- and [^{3}H]inositol-labeling, was thought to indicate the hydrolysis of PIP_2 by phospholipase C, formation of its water-soluble product, IP_3, was examined in [^{3}H]inositol-labeled mast cells treated with PMA for indicated intervals. Fig. 4 demonstrates an enhanced production of IP_3 in the mast cells treated with PMA for 5 min, as compared to the untreated control cells.

It has been a generally accepted idea that histamine release is caused by the synergistic effect of IP_3-induced Ca^{2+} mobilization and DG-activated protein kinase C, both of which are produced by phospholipase C-catalyzed hydrolysis of PIP_2. As previously reported by Katakami et al.(27), we could detect a small amount of histamine secreted (less than 30%) by stimulation with PMA alone at a concentration of 100 ng/ml (data not shown). Our present result indicated that the compound 48/80 challenge following the PMA-pretreatment caused repressed histamine secretion. Taken together with the above finding of substantial IP_3 generation, we are tempting to speculate that the histamine secretion suppressed

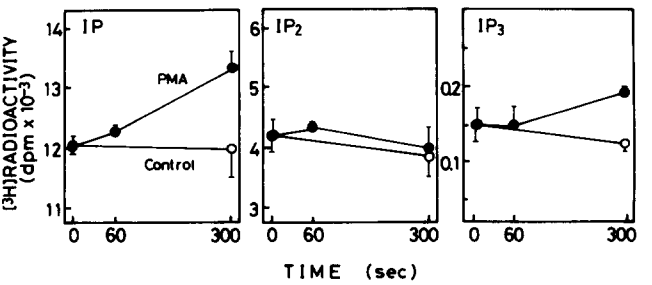

Fig. 4: Production of water-soluble inositol phosphates in PMA-treated mast cells. Rat peritoneal mast cells, prelabeled with [^{3}H]inositol, were stimulated with PMA (100 ng/ml) or DMSO (0.03%) for the indicated periods of time, and the reaction was terminated by adding 3.76 volumes of chloroform/methanol/conc. HCl (20/40/1, v/v). Water-soluble inositol phosphates were separated by chromatography on anion exchange resin as described in detail in "Materials and Methods". Results are expressed as the mean of duplicate determinations from a representative of two experiments showing similar data. Ranges refer to duplicate analysis.

by PMA may not be associated with IP_3 production. In fact, recent reports have shown the occurrence of secretory responses independent of IP_3 formation in ADP-activated platelets (28) and saponin-treated platelets stimulated with thrombin (29). Thus, it is postulated that compared to other types of cells where IP_3-induced Ca^{2+} mobilization is considered to be the crucial event in cellular responses, IP_3 may play a smaller role in triggering histamine secretion of mast cells. However, before drawing a conclusion, we should perform further extensive work including measurement of actual amount of formed IP_3.

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