

Inhibitory effect of adenine nucleotides and anti-allergic drugs on phosphorylation of phosphatidylinositol in rat mast cell granules

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Rat mast cell granules were obtained by sonication of highly purified rat mast cells and isolated in a Percoll gradient. Phosphorylation of endogenous phosphatidylinositol in rat mast cell granules, which is catalyzed by phosphatidylinositol kinase in the granules, was assayed by measuring the incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into phosphatidylinositol 4-phosphate. Lipids were isolated with methanol/chloroform/HCl and were separated by thin-layer chromatography on oxalic acid impregnated silica gel plates. Phosphatidylinositol 4-phosphate areas were identified by staining with iodine, scraped and measured for ^{32}P radioactivity. The phosphorylation reaction was inhibited by 50–500 μM adenosine, ADP and 500 μM AMP in a concentration-dependent manner. Among several anti-allergic drugs investigated, 100–1000 μM theophylline and 10–100 μM azelastine inhibited the phosphorylation reaction, but disodium cromoglycate and ketotifen had little effect.

Key words: adenine nucleotide; anti-allergic drugs; granule; mast cell; phosphatidylinositol.

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Release of mediators from mast cells occurs by exocytosis. During exocytosis granule membranes fuse with each other and with the mast cell plasma membranes, permitting the release of granules and their contents (17). However, little is known about the mechanisms of exocytosis from mast cells. Our studies have indicated that $^{32}\text{PO}_4$ incorporation into phosphatidylinositol 4-phosphate (referred to subsequently as diphosphoinositide [DPI]) and phosphatidylinositol 4,5-bisphosphate (triphosphoinositide [TPI]) was increased during mediator release from rat mast cells stimulated by concanavalin A (Con A) or compound 48/80 and these changes in the metabolism of polyphosphoinositides may be an intrinsic part of the biochemical mechanisms that control mediator release from mast cells (12). Control studies in the investigation in unstimulated rat mast cells

labelled with $^{32}\text{PO}_4$ suggested that at least 50% of total cellular radiolabelled polyphosphoinositide was in the granules (11, 12). Rat mast cell granules were shown to contain a highly active phosphatidylinositol (PI) kinase which catalyzes the formation of DPI from endogenous PI in the granule membrane (13). Interestingly, this enzyme was inhibited by cyclic AMP. Because intracellular increased level of cyclic AMP has been shown in activated mast cells (18), a possible role of cyclic AMP on the regulation of PI kinase in mast cell granules can be easily suggested. It has actually been demonstrated that in several cell systems, such as erythrocyte, neutrophil and hepatocyte, cyclic AMP inhibits PI kinases in the broken cells (4, 5, 9) and that treatment of intact cells with agents elevating cyclic AMP levels may inhibit PI kinase (9, 15). In the present report, effect of adenine nucleo-

tides on phosphorylation of PI in rat mast cell granules was studied. Evidence will be presented that they are able to inhibit the reaction. Moreover, some of anti-allergic drugs can actively inhibit it, suggesting that they may act through controlling PI turnover.

MATERIAL AND METHODS

Material

[$\gamma^{32}\text{P}$] ATP (4500 Ci/mmol) (ICN Pharmaceuticals, Irvine, CA, USA); Percoll (Pharmacia Co., Uppsala, Sweden); theophylline, adenosine, adenosine 3'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), trizma base, L- α -phosphatidylinositol, L- α -phosphatidylinositol-4-phosphate (diphosphoinositide [DPI]), L- α -phosphatidylinositol-4,5-bisphosphate (triphosphoinositide [TPI]), bovine serum albumin (BSA), 2-mercaptoethanol, sodium (tetra) ethylenediamine tetra acetate (EDTA), iodine, toluidine blue (Sigma Chemical Co., St. Louis, MO, USA); Liquifluor (New England Nuclear, Boston, MA, USA), silica gel plates (20 $\times$ 20 cm, 250 μm layer) (Macherey-Nagel, Duren, W. Germany); sodium dodecyl sulfate (Gallard-Schlesinger, Carle Place, NY, USA); acrylamide, NN'-methylene-bisacrylamide, ammonium persulfate, NNN'-N'-tetramethyl-ethylenediamine, bromophenol blue, Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA, USA); disodium cromoglycate (DSCG) (Fisons Pharmaceuticals, Loughborough, UK), ketotifen (Sandoz Pharmaceuticals, Basel, Switzerland), azelastine (Eisai Pharmaceuticals, Tokyo, Japan); oxalic acid, potassium oxalate, trichloroacetic acid (TCA) and others (Wako Pure Chemical Industries, Osaka, Japan).

Methods

Rat mast cells mixed with leukocytes and serosal cells were obtained by peritoneal and pleural lavage of male Sprague-Dawley rats (250 to 300

g; Shizuoka Laboratory Animals, Shizuoka, Japan) in amounts of about 1×10^6 mast cells per rat as described previously (13). The lavage medium was a heparinized (10 U/ml) balanced salt solution (BSS) (4.0 mM Na_2HPO_4 , 2.7 mM KH_2PO_4 , 150 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl_2 , pH 7.2) containing 0.046% (w/v) BSA. The mast cells in this mixture were purified on a Percoll gradient (6) and were more than 90% pure by toluidine blue staining reactions.

Intact membrane granules were isolated from sonicated rat mast cells on a Percoll gradient as described (10). Granules with intact membranes revealed by electron microscopy and ruthenium red staining (10) were used in the experiments after washing twice with BSS after the recovery from Percoll gradient.

To measure phosphorylating activity in isolated rat mast cell granules, incubations were carried out in conical polypropylene tubes in a total volume of 100 μl . Standard incubation mixtures contained 30 μl medium with 20 mM Tris/HCl, pH 7.4, 20 mM MgCl_2 , 10 μM [$\gamma^{32}\text{P}$]ATP (diluted with unlabelled ATP to 5×10^9 cpm/ μmol) mixed with 60 μl of resuspended mast cell granules in BSS and 10 μl of the drug solution. Each tube contained intact membrane granules from 1.5 to 2.3×10^6 mast cells, uncorrected for losses during purification. Based on direct protein measurements, this corresponds to approximately 15 to 16 μg granule protein/tube. In routine assays incubations were conducted at 30°C for 15 min and the reaction was stopped by the addition of organic solvents to the entire granule suspension as described below.

Lipids in the mast cell granules were isolated from granule suspensions by using a modified Bligh & Dyer (2) extraction procedure; 0.5 ml methanol, 0.25 ml chloroform and 0.1 ml 0.2N HCl were added directly to the reaction mixtures. Tubes were capped, shaken and centrifuged at $1000 \times g$ for 10 min. The upper aqueous phase was carefully removed by aspiration and aliquots of the organic phase were dried under N_2 gas at room temperature. Dried samples were dissolved in 60 μl chloroform and

stored at -80°C overnight. Phospholipids were separated by one-dimensional thin-layer chromatography (TLC) on silica gel plates as described previously (13). Before use, the plates were developed with either 1% (w/v) aqueous solution of oxalic acid or methanol/water (2:3 v/v) containing 1% (w/v) potassium oxalate. The oxalic acid or potassium oxalate-impregnated plates were dried at room temperature and activated at 110°C for 15 min. After the application of samples, oxalic acid-impregnated plates were developed with chloroform/methanol/4N NH_4OH (9:7:2, v/v/v); the potassium oxalate-impregnated plates with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, v/v/v/v/v). Lipid-containing areas were identified by staining with iodine, scraped into vials and measured for radioactivity in a liquid scintillation counter after addition of 10 ml of scintillation fluid (Liquifluor). The added unlabelled phosphoinositides were used both as internal standards and as isolated standards in an adjacent lane on the TLC plate. The radioactivities recovered in the DPI areas on oxalic acid- and potassium oxalate-impregnated silica gel plates in replicate samples corrected closely (less than 10% differences) with one another.

To verify that labelled DPI was indeed present when radioactivity was demonstrated in the DPI area, the presumed DPI spot on 1% (w/v) oxalic acid-impregnated plates was scraped into tubes and extracted a second time. A portion was measured for radioactivity and the remainder was chromatographed in the other TLC system on 1% (w/v) potassium oxalate-impregnated plates. Extraction was performed as described above except that 0.5 ml methanol, 0.25 ml chloroform and 0.25 ml 0.1 N HCl were used in the monophasic system and the extraction with chloroform in the two-phase system was repeated three times. After drying and autoradiography, the radioactivity on the second plate was in the DPI area. When samples were re-extracted by a more conventional extraction procedure for the major phospholipid species [involving the use of chloroform-methanol in the presence of 0.04% (w/v) CaCl_2 without dilute HCl], less than 1% of the

radioactivity was recovered. Conventional phospholipids, but not DPI or TPI, are recovered almost quantitatively in this extraction system.

Protein concentrations of granules were determined by the spectrometric method of Bradford (3), with crystalline BSA used as a standard.

Concentrations of granules were standardized with respect to one another on the basis of major granule membrane proteins. Granule proteins were separated by electrophoresis in the presence of sodium dodecyl sulfate in polyacrylamide slab gels, basically following the method of Laemmli (14). A linear gradient of polyacrylamide, from 6% (w/v) to 18% (w/v), was used as the separating gel with a 3% (w/v) acrylamide stacking gel. In all gels the ratio of acrylamide to bisacrylamide was 37:1 by weight. Samples were solubilized by heating for 5 min at 100°C in 70 μl of a mixture containing 6% (w/v) sodium dodecyl sulfate/30% (w/v) glycerol/15% (v/v) 2-mercaptoethanol/0.003% (w/v) bromphenol blue/0.75M Tris/HCl, pH 6.8. Gels were run at room temperature at 100 V and then fixed and stained in a solution containing 12% (w/v) TCA, 50% (v/v) methanol and 0.03% (w/v) Coomassie Brilliant Blue R-250. Gels were destained by washing several times with 7% (w/v) acetic acid/10% (v/v) methanol. Stained proteins were quantitated by scanning in a Zeineh Soft Laser scanning densitometer (Biomed Instruments, Chicago, IL, USA).

Analysis of data

Unless otherwise stated, each point illustrated in each figure represents the mean and standard error of the mean (SEM) of three experiments (each done in duplicate; differences between duplicates were less than 6%). Significance was determined by employing the independent Student t-test (two-tailed).

RESULTS AND DISCUSSION

50~500 μM adenosine decreased ^{32}P incorporation into DPI in rat mast cell granules in a

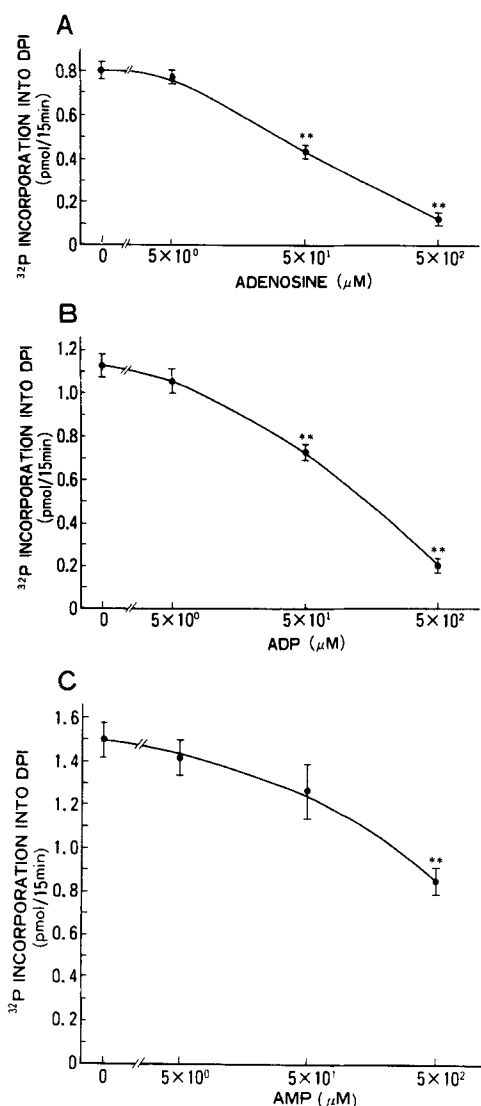


Fig. 1. Effects of adenine nucleotides on ^{32}P incorporation into DPI in rat mast cell granules. A: adenosine; B: ADP; C: AMP. Data represent the mean and standard error of the mean (SEM) of three experiments. **: statistical significance from the control at $P < 0.01$.

concentration-dependent manner. Per cent inhibition by 50 μM and 500 μM adenosine was 45.7% and 83.9% respectively. Also, 50~500 μM ADP and 500 μM AMP decreased DPI formation in rat mast cell granules in a concentration-dependent manner. ADP and AMP were weaker inhibitors than adenosine and %

inhibition by 50 μM and 500 μM ADP and 500 μM AMP was 34.8%, 81.7% and 43.1% respectively (Fig. 1). The experimental material was small and initial control values of ^{32}P incorporation were not the same in all experiments. It is therefore not possible to draw valid conclusions, but these results indicate that adenine analogue inhibited the phosphorylation of PI in rat mast cell granules. As shown previously (13), PI kinase is the enzyme which catalyzes this phosphorylation reaction in the granules. Even though the granules can contain other enzymes, it is easy to understand that adenine analogue inhibited the PI kinase in rat mast cell granules. Inhibitory effects of adenine analogue on the PI kinase in the granules were similar to that reported by other investigators (4, 5, 9) and these inhibitory effects on the enzyme activity in the granules can be due to competition with ATP for the enzyme site.

100~1000 μM theophylline also decreased ^{32}P incorporation into DPI in rat mast cell granules in a concentration-dependent manner and % inhibition by 100 μM and 1000 μM theophylline was 24.1% and 58.1% respectively. Among several anti-allergic drugs investigated, 1~100 μM DSCG and ketotifen had little effect on DPI formation in rat mast cell granules. However, 10~100 μM azelastine, [4-(p-chlorobenzyl)-2-(hexahydro-1-methyl-1H-azepine-4-yl)-1-(2H)-phthalazinone hydrochloride], decreased the ^{32}P incorporation into DPI in the granules in a concentration-dependent manner and % inhibition by 10 μM and 100 μM azelastine was 14.9% and 25.6% respectively (Fig. 2). Because theophylline is an adenine analogue, it is easy to understand that it inhibited the PI kinase activity due to competition with ATP for the enzyme site. On the other hand, azelastine is not an adenine analogue and there are no similarities between its structure and that of theophylline. Although, at this moment, there is uncertainty about its inhibitory mechanism on the PI kinase in rat mast cell granules, azelastine has been shown to inhibit immunologically and non-immunologically provoked histamine release from rat mast cells, and its action was several times more potent than those of the other anti-allergic drugs (7).

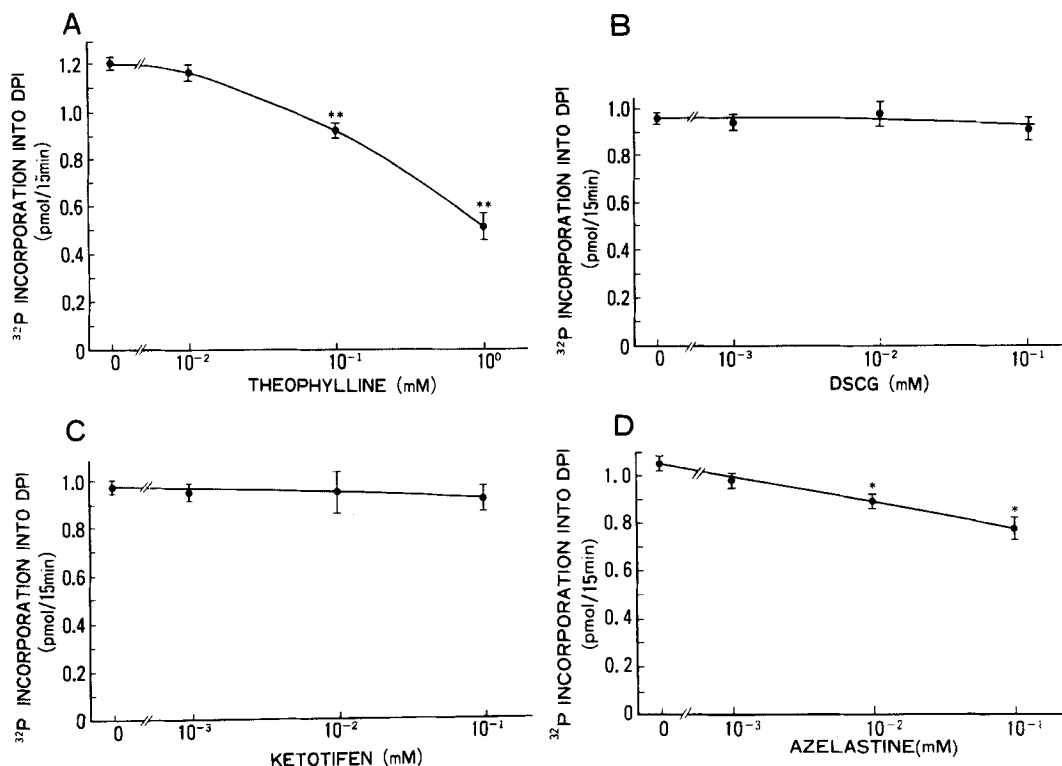


Fig. 2. Effects of theophylline, DSCG, ketotifen and azelastine on ^{32}P incorporation into DPI in rat mast cell granules. A: theophylline; B: DSCG; C: ketotifen; D: azelastine. Data represent the mean and standard error of the mean (SEM) of three experiments. * and **: statistical significance from the control at $P < 0.05$ and $P < 0.01$ respectively.

Theophylline is known to inhibit mediator release from rat mast cells through the alteration of the cellular cyclic AMP levels (8) and it has been suggested that some of the modes of action of azelastine may be a regulation of cyclic AMP levels in mast cells (1). Even then, the mechanism by which cyclic AMP regulate the mediator release from mast cells has not been demonstrated. Recently, much attention has been paid to cyclic AMP generation and phosphoinositide metabolism as an important intracellular biochemical event during receptor-mediated activation of many mammalian cells. Evidence is now accumulating that the former event may influence the latter; namely in some cells addition of cyclic AMP may result in decreased PI turnover (16). Present results raise an interesting possibility that some anti-allergic

drugs, such as theophylline and azelastine in this study, may inhibit mediator release from mast cells through controlling PI turnover with cyclic AMP generation; however, further studies are required for the conclusion because no measurement of cyclic AMP could be done in this study.

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