CALCIUM-DEPENDENT HISTAMINE RELEASE WITH DEGRANULATION FROM ISOLATED RAT MAST CELLS BY ADENOSINE 5'-TRIPHOSPHATE

Katsumi SUGIYAMA
Department of Pharmacology, Okayama University Medical School, Okayama

Received for publication October 26, 1970

It has generally been recognized that histamine release from mast cells induced by antigen-antibody reaction, as well as by certain basic compounds, is dependent upon the cellular energy generating processes since these reactions are inhibited by anoxic conditions and by metabolic inhibitors which uncouples oxidative phosphorylation or block respiratory enzymes (1-7). A preliminary study was attempted in order to see whether adenosine 5'-triphosphatase (ATPase) is really activated in association with the release of histamine. However, it was unexpectedly found, at the beginning of such a work, that adenosine 5'-triphosphate (ATP) itself caused a marked histamine release and this was accompanied with conspicuous morphological changes characterized by the degranulation of mast cells when the medium contained calcium ions.

Keller (8), and Diamant and Krüger (9, 10) have reported that histamine release was elicited by ATP present extracellularly, but the latter authors did not observe the occurrence of degranulation concomitant with the release of histamine even in the calcium-containing medium, unlike the present observation, although they found some morphological changes in mast cells discernible from those induced by a histamine liberator, compound 48/80.

The present paper describes observations on the morphological changes and on the release of histamine in isolated rat peritoneal mast cells under the effect of exogenous ATP, with special reference to the combined effect of calcium ions.

A preliminary report on these findings has been published earlier (11).

MATERIALS AND METHODS

Isolation of mast cells. Mast cells were isolated from the peritoneal fluid of Wistar rats (male, 250–350 g) by the gum arabic density gradient centrifugation procedure which is a modification of Kimura’s method (12).

Gum arabic stock solution was prepared as follows: A solution of 32 g of gum arabic powder dissolved in 90 ml of deionized water at 80°C was filtered through a gauze. After centrifugation to remove insoluble sediment, the solution was diluted with deionized water to make 1.090 in specific gravity, and pH adjusted to 7.4 with saturated NaOH. NaCl was added to give a final concentration 0.2%. This stock solution was kept at 4°C until use.
The rat was bled freely from the carotid arteries. Immediately after death, 10 ml of a buffered salt solution (Solution I) of the following composition, NaCl, 154 mm; KCl, 2.7 mm; CaCl₂, 0.9 mm; 10% (v/v) Sörensen phosphate buffer (Na₂HPO₄ and KH₂PO₄, 67 mm), pH 7.2, and 0.05% bovine serum albumin (BSA), was injected into the abdominal cavity. After gentle massage for 90 seconds of the gut through the abdominal wall the injected fluid was removed with a pipette, and centrifuged at 40 x g for 3 minutes. The pellet was dispersed in another buffered salt solution (Solution II) of the following composition, NaCl, 154 mm; Tris-HCl buffer, 10 mm (pH 7.3) and 0.05% BSA. This cell suspension was layered over two layers of gum arabic solution (the upper layer was made by diluting 0.7 ml of the stock solution with 0.35 ml of 0.9% NaCl, and the lower layer by diluting 0.8 ml of the stock solution with 0.2 ml of 0.9% NaCl), and then centrifuged at 650 x g for 10 minutes. Mast cells were precipitated in the bottom of the tube and other cells were concentrated in the interphase between two layers of gum arabic solution. Mast cells were removed, avoiding contamination with interface cells, and washed 3 times with the buffered salt solution to be used for incubation. All these isolation procedures were carried out at 4°C. The isolated mast cells responded normally to the histamine-releasing action of compound 48/80 or rabbit anti-rat serum (6). For most incubation experiments, Solution II was preferably used with or without Ca²⁺ unless otherwise stated because in either Solution I or II both histamine release and morphological changes were entirely the same depending on the presence or absence of Ca²⁺.

Experiments on histamine release. Mast cells (1-3 x 10⁶ cells/ml) were suspended in 2.3 ml of the incubation medium and prewarmed at 37°C for 5 or 10 minutes. Materials to be tested were added to give the total volume of 2.5 ml, and further incubation was made for another 10 minutes. When tested for the effect of inhibitors, mast cells were preincubated at 37°C for 10 minutes with inhibitors in a medium containing Ca²⁺ (10⁻⁴ M) before the addition of ATP (10⁻⁴ M).

Two milliliters of the reaction mixture was used for histamine assay and the remainder used for morphological studies. Tubes containing 2 ml of the mixture were centrifuged at 3,000 x g for 15 minutes, the supernatant was decanted, and the precipitate was added with 2 ml of 0.9% NaCl. Both supernatant and precipitate were added with 0.1 ml of 1 N HCl and boiled in a water bath for 3 minutes. After neutralization, histamine was assessed on atropinized guinea-pig ileum in Tyrode solution. All values were expressed as histamine base. The contraction of the guinea-pig ileum by histamine was unaffected in the presence of ATP below 10⁻³ M. If ATP was contained in higher concentration the sample was used after appropriate dilution since ATP itself otherwise caused contraction of the ileum.

Morphological observation under phase-contrast microscope. The mast cell suspension was maintained at 37°C in a small glass bath, made from a round-holed slide glass pasted with a cover glass on its under side, placed on a stage of an invert-type phase-contrast microscope (Olympus PMB, ×480), and morphological changes were observed or photographed.
Estimation of swelling of mast cells. The mast cells, fresh or fixed with 4% Formalin, were observed under an invert-type phase-contrast microscope. The size of fixed mast cells was determined by measuring the diameter of cells with a hemocytometer. Most of normal and ATP-treated mast cells were spherical although a few per cent of them were not. The average values were obtained from 30 cells of spherical form.

Measurement of time-course for histamine release and swelling of mast cells. Mast cells were pre-warmed at 37°C for 5 minutes in Solution II, then ATP was added (1 ml in total volume). For the experiment of histamine release, incubation medium contained Ca²⁺ (10⁻⁴ M), and the reaction was terminated at different time intervals by adding 9 ml of the chilled medium (0-4°C) and immediately transferring the tubes to an ice-cold water bath (13). For the experiment of swelling of mast cells, Ca²⁺ was omitted from the medium, and the reaction was stopped by the addition of Formalin (4%).

Preparation for electron microscopy. One milliliter of 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) was gently layered on the sediment obtained by centrifugation of a mixed cell suspension and left for 10 minutes at 4°C. The sediment then was removed and cut into 0.5 mm cubes to be fixed in 3% glutaraldehyde for 4 hours (4°C). The cubes were then washed in phosphate buffer (pH 7.2) for 12 hours, postfixed in a 1% solution of OsO₄ in phosphate buffer for 4 hours (4°C) and dehydrated in a series of ascending concentrations of ethanol and propylene oxide to be embedded in Epon 812. Sections were cut with glass knives on a Portar-Blum microtome, and stained with lead monoxide or in lead acetate, and observed with a HU-11 and a JEM-7 type electron microscope.

Chemicals. Nucleotides and nucleosides were obtained from the Sigma Chemical Co., U.S.A., gum arabic powder from the E. Merck AG, Darmstadt, and bovine serum albumin (Fraction V) from Armour Pharmaceutical Co., U.S.A.

RESULTS

General aspect of histamine release and gross morphological changes of mast cells by ATP and related compounds. When isolated mast cells were incubated in Solution I, the addition of ATP caused a marked histamine release. This was accompanied with certain morphological changes of the mast cells characterized by a marked swelling associated with evident extrusion of granules (degranulation), as described in details below. Both histamine release and degranulation reached the maximum when the concentration of ATP was 7.5 × 10⁻⁵ M, and declined with further increase in the concentration of ATP (Fig. 1). Such parallel increase of histamine release and degranulation by ATP seemed only to occur when the incubation medium contained Ca²⁺ because when mast cells were incubated with ATP in calcium-eliminated Solution I or Solution II neither histamine release nor degranulation could be observed and the only remarkable effect of ATP was a conspicuous swelling of the cells. Fig. 2 shows the actual measurement of the diameter of mast cells after their incubation with ATP of different concentrations in Solution II, along with histamine release which is below 10%, even in higher concentrations, 10⁻⁴ to 10⁻³ M, of ATP. The swelling is most remarkable at between 10⁻³ and 10⁻¹ M of ATP.
FIG. 1. Degranulation and histamine release from mast cells induced by ATP. Mast cells were incubated at 37°C for 10 minutes with ATP in Solution I (Ca²⁺ 9×10⁻⁷ M contained). •—•: Histamine release, ○—○: Degranulation (percentages of degranulated mast cells). Each plot based on mean value from 3 experiments.

FIG. 2. Effect of ATP on swelling of mast cells and histamine release, in the absence of Ca²⁺. Mast cells were incubated at 37°C for 10 minutes with ATP in Solution II, not containing CaCl₂. •: Mean diameter with standard errors of mean based on 30 cells of the same rat, ○: Histamine release, mean from 3 experiments.

Other nucleotides and nucleosides were tested for their effect on mast cells (Table I). Adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), adenosine 3', 5'-monophosphate (3', 5'-AMP), adenosine, adenine, and pyrophosphate were all ineffective in causing histamine release or morphological change of mast cells, regardless of the presence or absence of Ca²⁺ in the medium. Only 2'-deoxyadenosine 5'-triphosphate (dATP) showed a marked stimulating effect on histamine release which accompanies degranulation when the medium contained Ca²⁺. This compound also caused a marked swelling of mast cells in the Ca²⁺-absent medium. These effects were very similar and comparable to that of ATP. Inosine 5'-triphosphate (ITP), guanosine 5'-triphosphate
Table 1. Histamine release and swelling of mast cells by nucleotides, and related compounds. Mast cells were incubated with compounds to be tested at 37°C for 10 minutes in Solution II, with or without CaCl₂ (10⁻⁴ M). Percentages of histamine release are mean of 2-4 experiments, and swelling of mast cell is expressed as mean diameter of 30 cells from the same rat.

| Compounds   | Conc. (M) | Histamine release¹ | Swelling² (mean diameter, ± S.E.) |
|-------------|-----------|---------------------|-----------------------------------|
| None        | -         | 2.7                 | 12.2 ± 0.25                       |
| ATP         | 10⁻¹      | 67.4                | 19.8 ± 0.32                       |
|             | 10⁻³      | 6.4                 | 19.6 ± 0.22                       |
| ADP         | 10⁻⁴      | 7.6                 | 15.2 ± 0.43                       |
|             | 10⁻³      | 3.5                 | 12.1 ± 0.18                       |
| AMP         | 10⁻⁴      | 4.5                 | 12.4 ± 0.15                       |
| 3',5'-AMP   | 10⁻³      | 3.0                 | 12.1 ± 0.13                       |
| Adenosine   | 10⁻⁴      | 2.0                 | 12.5 ± 0.21                       |
| Adenine     | 10⁻⁴      | 1.8                 | 12.4 ± 0.30                       |
| Pyrophosphate | 10⁻⁴     | 1.5                 | 12.5 ± 0.16                       |
| d-ATP       | 10⁻¹      | 63.2                | 19.2 ± 0.28                       |
| GTP         | 10⁻¹      | 2.3                 | 12.3 ± 0.13                       |
| ITP         | 10⁻¹      | 2.6                 | 12.2 ± 0.20                       |
| CTP         | 10⁻¹      | 3.6                 | 12.0 ± 0.15                       |
| UTP         | 10⁻¹      | 2.5                 | 12.2 ± 0.16                       |

¹ In the presence of Ca²⁺.
² In the absence of Ca²⁺.

Fig. 3. Time-courses of histamine release by compound 48/80 and by ATP with Ca²⁺, and of swelling of mast cells by ATP alone. Mast cells were preincubated at 37°C for 5 minutes in Solution II, then added with compound 48/80 (0.5 µg/ml) or ATP (10⁻⁴ M). Total volume was 1 ml. For the experiments of histamine release incubation medium contained Ca²⁺ (10⁻⁴ M). The reactions were stopped by addition of 9 ml ice cold medium. For experiments of swelling of mast cells, Ca²⁺ was omitted from the medium and the reaction was terminated by addition of Formalin. ▲—▲ : Histamine release by compound 48/80 (mean of 3 experiments), ■ - • : Histamine release by ATP + Ca²⁺ (mean of 3 experiments), ○ - ○ : Diameter of mast cell (mean of 30 cells with S.E.).
(GTP), cytidine 5'-triphosphate (CTP), and uridine 5'-triphosphate (UTP) were all ineffective at any concentration up to 10^{-3} M.

**Time-course of histamine release and swelling of mast cells by ATP.** Time-course curves of both histamine release in the presence of Ca^{2+} (10^{-3} M) and swelling of mast cells in the absence of Ca^{2+} were followed at 37°C after the addition of ATP (10^{-4} M). As illustrated in Fig. 3, histamine release was first detectable 1 to 2 minutes after the addition of ATP and slowly increased until it was completed about 5 minutes after. This process was much slower than that of compound 48/80 which ended within 5 seconds or so. Swelling of the cells in the Ca^{2+}-absent medium, however, occurred rather rapidly. The original cell diameter (mean of 30 cells) of 12 μ increased to 16 μ after 15 seconds, wherefrom the ascending curve turned to a more linear slope reaching the maximum increase of 19.5 μ at about 4 minutes after contact with ATP.

**Effect of calcium.** The effect of concentration of CaCl₂ on the histamine release by ATP (10^{-4} M) is shown in Fig. 4. When CaCl₂ was present 5 minutes before the addition of ATP, the stimulating effect on histamine release appeared, reaching the maximum at around 10^{-4} M of CaCl₂, and then tended to become lower on further increase of CaCl₂ concentration. This lower effect by higher concentration of Ca^{2+} was reversible since the histamine release took place if these cells were washed and resuspended in the medium of lower Ca^{2+}-concentration before the addition of ATP. On the other hand, if CaCl₂ was added 5 minutes after the addition of ATP, the maximum effect of enhancing hista-
Histamine release was reached at $10^{-4}$ M of CaCl$_2$ and this effect was still observed at higher concentrations.

When mast cells were incubated with ATP in the absence of Ca$^{2+}$, histamine release was dependent upon the time when Ca$^{2+}$ was added. As illustrated in Fig. 5, if CaCl$_2$ was added after 5-minute incubation of mast cells with ATP in a Ca$^{2+}$-absent medium, histamine release was about 85% of the amount released when CaCl$_2$ was added just before ATP. Histamine release by the addition of CaCl$_2$ after 15-minute incubation with ATP was only 30% and such effect of CaCl$_2$ was abolished after a 20-minute incubation with ATP. In these cases the mast cells were already swollen by the effect of ATP before contact with Ca$^{2+}$. The enervated response of mast cells to Ca$^{2+}$ could no longer be restored by further addition of ATP.

Mast cells, which were swollen by preincubation with ATP for 5 minutes in the Ca$^{2+}$-absent medium, still had the activity of histamine release by the addition of CaCl$_2$. In this case, if ATP was washed out, histamine release from the mast cells could not be produced by the addition of CaCl$_2$, but in these washed cells, when both ATP and CaCl$_2$ were added simultaneously, histamine release did occur. GTP and ITP could not substitute for the action of ATP in this respect. This means that histamine releasing effect of ATP and Ca$^{2+}$ is not simply additive of the effects of individual agents.

The presence of EDTA in amount sufficient to chelate Ca$^{2+}$ completely inhibited histamine release by ATP and Ca$^{2+}$, but the swelling of mast cells appeared. Since EDTA itself had no ability to induce mast cell swelling, this observed swelling might be due to ATP after deprivation of Ca$^{2+}$. The swelling of mast cells induced by ATP ($10^{-5}$ M) was largely counteracted by the presence of Ca$^{2+}$ ($5 \times 10^{-4}$ M) previously added to the medium, but was not reversed by later addition of Ca$^{2+}$.

![Fig. 5. Effect of Ca$^{2+}$ on histamine release by ATP in relation to the time of addition of CaCl$_2$. Mast cells were incubated in Solution II. CaCl$_2$ ($10^{-4}$ M) was added at different periods of time after exposure to ATP ($10^{-4}$ M). ○—○: Histamine release due to ATP ($10^{-4}$ M) alone added at zero time of incubation, ●—●: Histamine release stimulated by the addition of Ca$^{2+}$ at indicated time of incubation with ATP. Each plot based on duplicate experiments. Spontaneous histamine release deducted.](image-url)
Effect of magnesium. The histamine release induced by ATP in the presence of Ca$^{2+}$ was inhibited by Mg$^{2+}$. The extent of this inhibition was dependent on the concentration of Mg$^{2+}$ relative to that of Ca$^{2+}$, suggesting the existence of a competition between Ca$^{2+}$ and Mg$^{2+}$ in the histamine release by ATP (Fig. 6). On the other hand, the swelling of mast cells induced by ATP ($10^{-4}$ M) was prevented by the presence of Mg$^{2+}$ in concentrations higher than $5 \times 10^{-4}$ M, as was the case by Ca$^{2+}$.

Effect of other metal ions. The effect of various metal ions on the histamine release by ATP is shown in Table 2. Sr$^{2+}$ and Ba$^{2+}$ were less effective than Ca$^{2+}$, and other cations

![Fig. 6. Effect of Mg$^{2+}$ on histamine release by ATP. Mast cells were incubated with ATP ($10^{-4}$ M) at 37°C for 10 minutes in Solution II. Mg$^{2+}$ has no enhancing effect on the histamine release by ATP, but exerts inhibition on the enhancing effect of Ca$^{2+}$ when the concentration of Mg$^{2+}$ exceeds that of Ca$^{2+}$. Each plot from duplicate experiments.](image)

| Metal salts | Conc. (M) | Histamine release (%) |
|-------------|-----------|-----------------------|
| None        | —         | 13.2                  |
| KCl         | $3 \times 10^{-4}$ | 12.2                  |
|             | $1.5 \times 10^{-3}$ | 3.4                  |
| CaCl$_2$    | $1 \times 10^{-4}$ | 74.3                  |
|             | $5 \times 10^{-4}$ | 70.3                  |
| MgCl$_2$    | $1 \times 10^{-4}$ | 15.7                  |
|             | $5 \times 10^{-4}$ | 8.8                   |
| SrCl$_2$    | $1 \times 10^{-4}$ | 24.7                  |
|             | $5 \times 10^{-4}$ | 27.8                  |
| BaCl$_2$    | $1 \times 10^{-4}$ | 27.7                  |
|             | $5 \times 10^{-4}$ | 27.0                  |
| ZnCl$_2$    | $1 \times 10^{-4}$ | 2.4                   |
|             | $5 \times 10^{-4}$ | 3.7                   |
| MnCl$_2$    | $1 \times 10^{-4}$ | 14.8                  |
|             | $5 \times 10^{-4}$ | 7.6                   |
Fig. 7. Effect of pH on histamine release and swelling of mast cells by ATP. Mast cells were preincubated at 37°C for 5 minutes in the medium containing NaCl (154 mm), 0.05% BSA, and 10 mm Tris-maleate buffer, then ATP (10^-6 M) was added and further incubation was made for 10 minutes. Incubation medium contained Ca^2+ (10^-4 M) for the experiment on histamine release but not for the experiment on swelling of mast cells. •-○: Histamine release in the presence of ATP, ○—○: Histamine release in the absence of ATP (mean of 3 experiments), •--•: Diameter of mast cell in the presence of ATP, •--•: Diameter of mast cell in the absence of ATP (mean of 30 cells with S.E.).

Fig. 8. Effect of temperature on histamine release and swelling of mast cells by ATP. Mast cells were preincubated at 37°C for 5 minutes in Solution II, then ATP (10^-6 M) was added and further incubation was made for 10 minutes. Incubation medium contained Ca^2+ (10^-4 M) for the experiment on histamine release but not for the experiment on swelling of mast cells. •—○: Histamine release in the presence of ATP, ○—○: Histamine release in the absence of ATP (mean of 3 experiments), •--•: Diameter of mast cell in the presence of ATP, •--•: Diameter in the absence of ATP (mean of 30 cells with S.E.).
such as K⁺, Mg⁺, Zn²⁺, and Mn²⁺ were all ineffective in enhancing the histamine release by ATP.

Effect of pH. The pH of Solution II was variably adjusted with 10 mM Tris-maleate, instead of Tris-HCl. In the presence of Ca²⁺, the pH optimum for the ATP-induced histamine release was 7 and the release was largely depressed at pH's lower than 6.5 or higher than 8. On the other hand, in the Ca²⁺-absent medium, the curve for the swelling action of ATP reached the maximum at pH 7.5 but this shifted to a plateau on the alkaline side (Fig. 7).

Effect of temperature. Histamine release by ATP in the presence of Ca²⁺ was highly sensitive to the temperature change (Fig. 8). The optimum temperature was 37°C. At the temperature below 5°C and above 42°C, histamine release was inhibited. The reaction was irreversibly inhibited when the mast cells were preincubated at 42°C for 1 minute whereas the inhibition by the low temperature was reversible. On the other hand, in the absence of Ca²⁺ the cellular swelling by ATP was prominent at between 30°C and 45°C but disappeared suddenly at 50°C. ATP could still cause the swelling of mast cells even after they were preincubated at 45°C for 5 minutes.

Effect of metabolic inhibitors and of other nucleotides. Table 3 shows the effect of some metabolic inhibitors on the release of histamine induced by ATP and Ca²⁺. A marked inhibition was exhibited by SH-inhibitors such as N-ethylmaleimide and p-chloromercuribenzoate while iodoacetate was less effective. Cyanide and 2, 4-dinitrophenol were also effective inhibitors, but little inhibition was exerted by sodium fluoride. Inhibition by some heavy metal ions, Zn²⁺, Cu²⁺, Mn²⁺ and Fe³⁺, was noted. Ouabain was not effective. Cocaine, which is known to be a stabilizer for the cell membrane (14), revealed only a weak inhibition. On the other hand, in the Ca²⁺-absent medium, the swelling of
mast cells induced by ATP was not inhibited by the presence of any of these inhibitors in concentrations causing inhibition of the histamine release.

The presence of other nucleotides, ADP, AMP, ITP, and GTP (10^{-4} M) did not influence the histamine release by ATP (10^{-4} M) in the presence of Ca^{2+} (10^{-4} M) nor interfered with the cellular swelling by ATP (10^{-4} M) in the absence of Ca^{2+}.

*Morphology of mast cells. A. Phase-contrast microscopic observation.* When ATP was added to give a concentration of 10^{-4} M, in a Ca^{2+} (10^{-4} M)-containing medium, mast cells became swollen 5 to 10 seconds later, and small vacuoles were formed successively around the granules and fused with each other. These changes occurred in successively increasing number of cells. During these events in the cytoplasm, remarkable alterations were observed in the granules; the majority of intracellular granules enlarged and their dark contrast diminished and became blurred. Along with these changes, conspicuous extrusion of granules out of the margin of the cell occurred, first one by one at intervals of a few seconds, but soon this became faster in continuous succession all over the cell surface. Some of the extruded granules adhered on the cell surface but others in large portion were dispersed and drifted in the surrounding medium. Degranulated mast cells were easily distinguishable from those unaffected, usually existing in a few per cent, by

![Fig. 9. Phase-contrast micrographs of isolated rat peritoneal mast cells. 470.](image-url)

A : Control mast cells incubated in Solution II (Ca^{2+} contained). B : Swollen mast cells 10 minutes after exposure to ATP (10^{-4} M) in the absence of Ca^{2+}. Histamine release from these cells was below 10%. C : Mast cell changes 10 minutes after contact with ATP (10^{-4} M) in the presence of Ca^{2+} (10^{-4} M). Histamine release was 65-85%. D : Mast cell changes 3 minutes after exposure to compound 48-80 (0.5 μg ml).
the adherance of extruded granules on the cell membrane and also by a peculiar appearance of cytoplasm (Fig. 9, C). The time elapsing from the beginning of swelling to completion of the degranulation varied from cell to cell but the average was between 3 and 5 minutes; therefore, the whole reaction was rather sluggish.

These morphological changes induced by ATP were similar to those by compound 48/80 as far as the fundamental features of granule extrusion and of vacuole formation are concerned but there were some differences between the two cases in general appearance. The reactions by ATP required a longer lag phase and was much more sluggish than those by compound 48/80. Under the effect of compound 48/80, degranulated mast cells showed a mulberry-like appearance by the adherance of extruded granules on the cell membrane (Fig. 9, D), while in those affected by ATP, a majority of extruded granules were dispersed in the medium. Therefore, contour of the nucleus was clearly visible in ATP-treated mast cells, whereas this was uncommon in the mast cells treated with compound 48/80. Another difference was the formation of large blisters frequently met in ATP-treated mast cells, which was rare in the compound 48/80 cases (Fig. 9, C, D). These patterns of morphological changes induced by ATP bear a close resemblance to the ones observed in the mast cells treated with rabbit anti-rat serum, reported by Sacki (6).

Fig. 10. Electron micrographs of rat mast cell. 6,800.
A: A normal mast cell of the peritoneal fluid of rat. B: A mast cell treated with ATP (10^{-1}M) in the Ca^{2+}-absent medium. The mast cell is significantly swollen, and microvilli disappear. No cytoplasmic vacuolation is seen despite of marked widening of intergranule spaces. Granules are not different from the normal mast cell in size, shape, and in electron density.
In other experiments, incubation medium was devoid of Ca\(^{++}\). On addition of ATP nearly all mast cells became swollen in 5-10 seconds. There was a widening of the intergranular spaces, but the size and other appearance of granules remained unchanged. Therefore, each granule was more clearly visible in rather lucid cytoplasm and the Brownian movement of granules could be seen. Despite little change of the granules, swelling of the nucleus was remarkable (Fig. 9, B).

**B. Electron microscopic observation.** The appearance of normal mast cells were not different from the reports of other workers (15-20) (Fig. 10, A). After the treatment with ATP (10\(^{-4}\) M) in the presence of Ca\(^{++}\) (10\(^{-4}\) M), the mast cell appeared profoundly altered (Fig. 11). Granules lay in the electron-lucent material (vacuoli) which was formed.

![Fig. 11. Electron micrograph of rat mast cell treated with ATP (10\(^{-4}\) M) in the presence of Ca\(^{++}\) (10\(^{-4}\) M). 14,000. The cell membrane disintegrated and the cytoplasm filled with many large vacuoles containing altered granules, one of which appears outside the cell. See text.](image-url)
by widening of the perigranular spaces; perigranular membranes widely separated from
the granule formed a continuous membrane delimiting the cytoplasm which now appeared
to be not uniformly distributed but retracted around the perinuclear region. Some of
the cytoplasmic matrix constituted the peripheral border of the cell and connected, with
slender cytoplasmic processes, to the cytoplasmic portion of the perinuclear region. The
original perigranular membrane which now formed the limiting wall of the vacuoles was
in close to the plasma membrane and a thin cytoplasmic layer was sandwiched between
these two membranes. The septa, formed of cytoplasm, between vacuoles were broken
at many places, this enabling many vacuoles to fuse with each other forming a large pool.
Borders and broken ends of the cytoplasmic septa were not distinctly defined but rather
blurred, suggesting occurrence of dissolutive changes.

Changes in the granules were also remarkable. All granules lying in the vacuoles
were swollen to various degrees and general electron density was decreased; the granule
matrix showed a substructure appearing as an extremely fine filamentous or reticular mesh-
work in which small electron-dense particles were visualized. Some of coarse and low-
density granules were partially dissolved leaving only traces of loose, wide-meshed material.
Some altered granules were melted together and sometimes numerous fine filaments inter-
connected the granules.

All these changes of mast cells may not essentially be different from those of com-
pound 48/80-treated mast cells (20), but on comparing in detail the effects of these two
releasers, which were observed using the same technique in this laboratory (20), the differ-
ence seemed to be a more frequent or predominant occurrence of dissolutive changes in
the ATP-Ca\textsuperscript{2+} effect than in the effect of compound 48/80.

On the other hand, the mast cells affected by ATP in the absence of Ca\textsuperscript{2+} revealed
a quite different feature of changes (Fig. 10, B). All the mast cells were round and swol-
len nearly twice in diameter as normal cells. The nucleus was also remarkably enlarged
and all showed a rounded shape while they mostly were much more deformed in normal
cells. Larger portion of the nucleus was of low electron density although further details
of the structural changes can not be mentioned. In these cells intergranule spaces were
evidently widened but these spaces were filled not with vacuoles but with cytoplasmic ma-
trix. Granules were surrounded closely with perigranular membrane as in normal state
without leaving electron-lucent spaces. Most granules were not different from normal
in size, shape, electron density, and other aspects. Outer border of the cell clearly retained
its continuity although villous protrusion dissapeared: there were no extruding granules
out of the margine of the cell.

DISCUSSION

Exogenous ATP produced a marked histamine release accompanied by degranulation
from rat peritoneal mast cells when the medium contained calcium ions, while in the ab-
sence of calcium, ATP produced conspicuous swelling of mast cells but neither substantial
histamine release nor degranulation. Diamant and Krüger (9, 10), who reported a stimu-
lating effect of ATP in histamine release from isolated rat mast cells, did not observe de-
granulation in the Ca\textsuperscript{2+}-containing medium. They used mast cells isolated with Ficoll
while gum arabic was used in the present experiment. The discrepancy may not be due to
the difference in isolation procedure since similar results could have been obtained either
with Ficoll-isolated mast cells or with mast cells in mixed peritoneal cells (unpublished
observation).

ATP-induced morphological changes of mast cells were quite different in the presence
and absence of Ca\textsuperscript{2+}. In the presence of Ca\textsuperscript{2+}, conspicuous alterations occurred in the gran-
ules, such as marked swelling, lowered density, and substructural changes, and cytoplasmic
vacuolation which developed from widening of perigranular spaces was also a prominent
characteristic feature. On the other hand, in the absence of Ca\textsuperscript{2+}, characteristic feature
of the change in mast cells was a significant increase in cell volume, 1.5 to 2-fold enlarge-
ment in diameter, but in these cells granules showed normal size and appearance, and
perigranular membrane closely surrounded the surface of granules without leaving a space.
These findings favor the view that histamine release is intimately connected with the for-
mation of vacuoles which may probably be the necessary prerequisite for the occurrence
of structural changes in granules (20).

The mechanism of action of ATP in the cellular swelling is unknown. Since the cel-
luar swelling had little dependency on pH, since it could not be inhibited by ouabain
and since, in my recent experiment (21), ATP-hydrolysing activity of mast cells could not
be seen, the membrane ATPase connected with ionic pump may not be concerned much
in this mechanism. Stewart et al. (22) reported a similar phenomenon with TA\textsubscript{a} ascites
tumor cells. These tumor cells were also extremely increased in volume by exogenous
ATP in Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free medium, and this increase was reversible upon addition of
Ca\textsuperscript{2+} or Mg\textsuperscript{2+} to the media. Experimental evidence suggested that this phenomenon may
be due to an alteration in membrane permeability. The binding of ATP to the mast
cell membrane may produce alteration of membrane lipoprotein substructure leading to
changes in membrane permeability and enabling the entry of extracellular ions and water
which induce cellular swelling. However, such an increase in permeation of extracellular
ions through the cell membrane may not trigger the activation of enzymic processes
necessary for histamine release. Tasaka et al. (23, 24) introduced a relatively high con-
centration of Ca\textsuperscript{2+} or Na\textsuperscript{+} into inside of rat mast cell by iontophoresis. The mast cell
was evidently swollen but neither degranulation nor release of histamine was evoked.

The action of ATP on mast cells in Ca\textsuperscript{2+}-containing medium resembled that of com-
ound 48/80, but there were some differences between these two types of histamine re-
leaser. 1) The action of ATP is dependent on Ca\textsuperscript{2+} whereas that of 48/80 is not, as far
as in vitro experiment is concerned. 2) Time-course of histamine release by ATP-Ca\textsuperscript{2+}
is much slower than that by 48/80. 3) There are some differences in morphological change
of mast cells. In ATP-Ca\textsuperscript{2+}-treated mast cells, degranulated granules are less adhesive
to the surface of cell membrane and a larger part of extruded granules are dispersed, drift-
ing in the surrounding medium, whereas in 48/80-degranulated mast cells much granules
adhere on the surface membrane showing more mulberry-like appearance. Blister formation is much more frequent in ATP-Ca⁺⁺-treated mast cells. Electron microscopic findings suggested more frequent occurrence of dissolutive changes in ATP-Ca⁺⁺-treated mast cells. These characteristic features in the action of ATP-Ca⁺⁺ indicate that the action of ATP on mast cells bear resemblance to those of rabbit antiserum (6, 20) or α-chymotrypsin (6).

Our recent work (23, 25) revealed that when ATP was applied iontophoretically close to the surface membrane of mast cell with a micropipette in Ca⁺⁺-containing medium, a local degranulation occurred as was observed with compound 48/80. Moreover, the application of Ca⁺⁺ by micropipette on a mast cell pretreated with ATP in Ca⁺⁺-free medium similarly produced a local degranulation on the cell surface. These observations suggest that the interaction of ATP-calcium-membrane subunit may activate the intracellular processes leading to degranulation and histamine release, first locally near the membrane site of application. The importance of cell membrane would be stressed also in this action since intracellularly ejected ATP did not produce degranulation even in Ca⁺⁺-containing medium.

Histamine release by ATP with Ca⁺⁺ was dependent on pH and temperature, and irreversibly inhibited by the treatment at 42°C for 1 minute. It was also inhibited by SH-inhibitors, uncoupling agents of oxidative phosphorylation, and also by heavy metal ions. These observations are similar to the action of many other histamine releasers and histamine release in antigen-antibody reaction, and indicate that this action depends on the functioning state of mast cell or its membrane and the process involves heat-labile factor which has not been identified. The simple swelling by ATP in the absence of Ca⁺⁺ was not effected in alkaline pH and inhibited at 50°C, but not at 42°C, and not affected by the mentioned metabolic inhibitors. Therefore, cellular swelling and histamine release are independent processes. It seems improbable that the exogenous ATP is utilized as energy source for the histamine release processes since Ca⁺⁺-activated ATPase was not detected in the intact mast cells as will be reported later (21).

The presence of extracellular calcium ions was shown to be necessary for secretions in other tissues, such as secretion of catecholamines from adrenal chromaffin cells (26), acetylcholine from nerve endings (27), secretion of protein from submaxillary salivary gland (28), and protein release from leucocidine-treated leucocytes (29). Therefore, it is tempting to speculate as to whether a mechanism common with secretion processes in these tissues is involved in the process of histamine release although available evidence is still lacking for decisive conclusion. In this connection, it is interesting to note the antagonism between Ca⁺⁺ and Mg⁺⁺ in histamine release by ATP as in the case of catecholamine secretion (30) and protein release from leucocytes (31).

SUMMARY

1. Histamine release accompanied with degranulation was induced in isolated rat peritoneal mast cells by ATP when Ca⁺⁺ was present in the medium, while in Ca⁺⁺-free
medium mast cells swelled significantly but there could be observed neither degranulation nor substantial release of histamine.

2. These phenomena were similarly observed with dATP, but ITP, GTP, UTP, ADP, AMP, 3', 5'-AMP, adenosine, adenine, and pyrophosphate were all ineffective, irrespectively of the presence or absence of Ca²⁺.

3. Sr²⁺ and Ba²⁺ could substitute for Ca²⁺ in stimulating histamine release by ATP, although much less effective. K⁺, Mn²⁺, Mg²⁺, Fe²⁺, and Zn²⁺ were ineffective. The effect of Ca²⁺ was quantitatively inhibited by Mg²⁺. High concentration of Ca²⁺ inhibited histamine release by ATP. EDTA inhibited the histamine release by ATP with Ca²⁺ but not the cellular swelling by ATP in the absence of Ca²⁺.

4. The pH optimum was 7 and temperature optimum was 37°C for the histamine release by ATP with Ca²⁺. The histamine release was irreversibly inhibited by treatment at 42°C for 1 minute, while the cellular swelling induced by ATP in the absence of Ca²⁺ was inhibited at 50°C. KCN, DNP, SH-inhibitors, and heavy metal ions inhibited the histamine release, but ouabain and cocaine were virtually ineffective in inhibiting the histamine release.

5. Time-course of histamine release by ATP with Ca²⁺ was much slower than that by compound 48/80, requiring 5 minutes for completion.

6. Light and electron microscopic observations revealed certain differences in morphological changes of mast cells between the effects of ATP with Ca²⁺ and of compound 48/80, although vacuolation and structural alteration of granules were prominent in both. Mast cells swollen by ATP in Ca²⁺-free medium contained apparently normal granules and were devoid of vacuoles.

7. The mechanism of histamine release by ATP was discussed.

Acknowledgement: I wish to thank Prof. H. Yamasaki for his guidance and criticism in this work. Acknowledgement is also due to Dr. Y. Ohara for preparation of the electron micrographs.

REFERENCES

1) Mongar, J.L. and Schild, H.O.: Physiol. Rev. 42, 226 (1962)
2) Moussatché, H. and Prouvost-Danon, A.: Experientia 14, 414 (1958)
3) Yamasaki, H., Muraoka, S. and Endo, K.: Jap. J. Pharmac. 10, 21 (1960)
4) Diamant, B.: Acta physiol. scand. 55, 11 (1962)
5) Diamant, B. and Uvnás, B.: Acta physiol. scand. 53, 315 (1961)
6) Saeki, K.: Jap. J. Pharmac. 14, 375 (1964)
7) Yamasaki, H. and Saeki, K.: Proc. Japan Acad. 41, 958 (1965)
8) Keller, R.: Tissue Mast Cells in Immune Reactions, pp. 38-39, S. Kargar, Basel (1966)
9) Diamant, B. and Krüger, P.G.: Acta physiol. scand. 71, 291 (1967)
10) Diamant, B. and Krüger, P.G.: J. Histochem. Cytochem. 16, 707 (1968)
11) Sugiyama, K. and Yamasaki, H.: Jap. J. Pharmac. 19, 175 (1969)
12) Kimura, E.: Protein-Nucleic Acid-Enzyme 12, 338 (1967)
13) Bloom, G.D., Fredholm, B. and Haegeermark, Ö.: Acta physiol. scand. 71, 270 (1967)
14) Shanes, A.M.: *Pharmac. Rev.* **10**, 59 (1958)
15) Smith, D.E. and Lewis, Y.S.: *J. biophys. biochem. Cytol.* **3**, 9 (1957)
16) Bloom, G., Larsson, B. and Smith, D.E.: *Acta path. microbiol. scand.* **40**, 309 (1957)
17) Horsfield, G.I.: *J. Path. Bact.* **90**, 599 (1965)
18) Bloom, G.D. and Hægermark, Ö.: *Expl. Cell. Res.* **40**, 637 (1965)
19) Singletone, E.M. and Clark, Jr., S.L.: *Lab. Invest.* **14**, 1744 (1965)
20) Yamasaki, H., Fujita, T., Ohara, Y. and Komoto, S.: *Arch. histol. jap.* **31**, 393 (1970)
21) Sugiyama, K.: *Jap. J. Pharmac.* (1971) (in press)
22) Stewart, C.C., Gasic, G. and Hempling, H.G.: *J. Cell. Physiol.* **73**, 125 (1969)
23) Tasaka, K., Sugiyama, K., Komoto, S. and Yamasaki, H.: *Proc. Japan Acad.* **46**, 317 (1970)
24) Tasaka, K., Endo, K. and Yamasaki, H.: *Proc. Japan Acad.* **46**, 831 (1970)
25) Tasaka, K., Sugiyama, K., Komoto, S. and Yamasaki, H.: *Proc. Japan Acad.* **46**, 311 (1970)
26) Douglas, W.W. and Rubin, R.P.: *J. Physiol.* **159**, 40 (1961)
27) Katz, B.: *Proc. R. Soc. B.* **155**, 455 (1962)
28) Douglas, W.W. and Poisner, A.M.: *Nature, Lond.* **196**, 379 (1962)
29) Woodin, A.M. and Wieneke, A.A.: *Biochem. J.* **87**, 487 (1963)
30) Douglas, W.W. and Rubin, R.P.: *J. Physiol.* **167**, 288 (1963)
31) Woodin, A.M. and Wieneke, A.A.: *Biochem. J.* **90**, 498 (1964)