Biochemical Pharmacology, Vol. 25, pp. 1907–1913. Pergamon Press, 1976. Printed in Great Britain.

INHIBITORY ROLE OF CYCLIC ADENOSINE 3′5′-MONOPHOSPHATE IN HISTAMINE RELEASE FROM RAT PERITONEAL MAST CELLS *IN VITRO*

HIDEYA HAYASHI, ATSUSHI ICHIKAWA, TERUMI SAITO and KENKICHI TOMITA
Department of Health Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan 606

(*Received* 9 *September* 1975; *accepted* 2 *January* 1976)

Abstract—Cyclic adenosine 3′,5′-monophosphate (cyclic AMP) accumulated in rat peritoneal mast cells during incubation with epinephrine and theophylline, correlating well with the inhibition of histamine release from the cells by these agents, but occurring on different time courses. During stimulation with epinephrine, the initial, rapid accumulation of radioactive cyclic AMP in mast cells previously labeled with radioactive adenine preceded the elevation of total cyclic AMP content and the increase in the inhibition of histamine release. Theophylline, on the other hand, rapidly elevated the cellular levels of cyclic AMP before the accumulation of radioactive cyclic AMP and the inhibition of histamine release became detectable. Cyclic AMP, dibutyryl cyclic AMP, epinephrine and theophylline were all effective in suppressing histamine release from mast cells and from isolated granules in isotonic sucrose solution, but were less effective or not effective in NaCl-containing media. Cyclic AMP also suppressed the extrusion of granules from mast cells induced by compound 48/80 in isotonic sucrose solution. These results indicate that the formation of cyclic AMP from special pools of ATP is required for inhibiting histamine release from mast cells, and also that cyclic AMP regulates histamine release by interfering in an early stage of the release process prior to the interaction of the granular amine with the extracellular cations.

Accumulated indirect evidence indicates that cyclic adenosine 3′,5′-monophosphate (cyclic AMP) plays a regulatory role in the release of histamine from mast cells as well as from certain histamine-containing tissues and cells.

Cyclic AMP, its N^6,$O^{2'}$-dibutyryl derivative (dibutyryl cyclic AMP), or agents known to increase the cellular levels of cyclic AMP suppressed the release of histamine from mast cells which had been induced by various amine liberators such as ATP [1], inorganic pyrophosphate [1] and compound 48/80 [2, 3]. These suppressors also inhibited immunological histamine release from rat mast cells [2, 4] as well as from human leukocytes [5–7] and human lung tissue [8–10]. However, the manner in which this nucleotide regulates histamine release is not entirely clear yet.

As to the amine-releasing mechanism, Uvnäs *et al.* [11–14] have proposed that the amine release induced by degranulating agents is a two-stage process: a primary exposure of granules to the extracellular environment either by extrusion of granules or through the newly formed intracellular cavities communicating with the external space; and a secondary ionic exchange between the granular amines and the extracellular cations.

In order to define the role of the adenyl cyclase system in histamine release, we evaluated the effect of several agents on the accumulation of cyclic AMP in mast cells, and also examined whether cyclic AMP exerts its inhibitory effect by affecting the primary degranulation step (step I) or the secondary ionic exchange step (step II) during histamine release.

The present report describes the stimulatory effects of epinephrine and theophylline on the accumulation of cyclic AMP in isolated rat peritoneal mast cells, and also the major inhibitory effect of the nucleotide on the primary degranulation step during histamine release induced by compound 48/80.

METHODS AND MATERIALS

Preparation of mast cells. Mast cells were isolated from peritoneal cavity fluids of normal Wistar rats weighing 250–350 g using a slight modification of the method described by Chakravarty and Zeuthen [15]. The cells were suspended in the ice-cold phosphate-buffered saline (PBS) of Dulbecco and Vogt [16] containing heparin (0.3 mg/ml) and adjusted to 20% with Ficoll, and layered on 30 and 40% Ficoll in a siliconized glass centrifuge tube. After centrifuging at 400 *g* and 4° for 15 min, the layer containing mast cells was pipetted out, washed three times with 5 ml PBS, and suspended in the same medium at 10^5 to 10^6 cells/ml. The cell suspensions contained 90 per cent or more viable mast cells, as determined by the toluidine blue (0.1% in 50% ethanol) staining test of Bray and VanArsdel [17], and by the nigrosin (0.2% in Hank's solution) staining viability test of Kaltenbach *et al.* [18].

Isolation of mast cell granules. Instead of pretreating mast cells with distilled water or with compound 48/80 [19], the cells were homogenized in ice-cold 0.25 M sucrose containing 1 mM Tris–HCl (pH 8.0) (1 to 5 × 10^7 cells/0.5 ml), and the granules were isolated by centrifuging at 2700 *g* for 15 min after the initial centrifuging at 110 *g* for 3 min [11].

Incubation of mast cells and estimation of histamine. Mast cells (10^5 to 10^6 cells) suspended in 1 ml PBS were preincubated with various concentrations of the substances to be tested at 37° for 15 min, and then

for another 5 min after the addition of compound 48/80 (1 μg/ml). The reaction was terminated by cooling the mixture to ice-cold. After centrifuging at 500 *g* for 5 min, histamine in the supernatant fluids was assayed fluorometrically using the method of Shore *et al.* [20]. The total content of histamine in intact cells was the amount of the amine extracted from the cells which had been frozen and thawed five times [1]. The concentration of histamine released from mast cells by compound 48/80 alone was taken as 100%, and the per cent inhibition of release by each drug was calculated on that basis.

Measurement of total cyclic AMP. An aliquot (0.5 ml) of mast cell suspension was sedimented by centrifugation at 500 *g* for 5 min, mixed with 50% perchloric acid (50 μl), freeze-thawed ten times, and centrifuged at 500 *g* for 10 min. The supernatant fluid was neutralized with 2 M $KHCO_3$, evaporated to dryness *in vacuo*, and dissolved in a small amount of distilled water.

Extracted cyclic AMP was determined using a protein-binding method of Gilman [21], employing protein kinase of rabbit muscle purified according to Miyamoto *et al.* [22] up to the stage of DEAE-cellulose chromatography.

Measurement of cyclic AMP accumulation. A method applied to adipocytes by Kuo and De Renzo [23] and to leukocytes by Bourne *et al.* [24] was adapted to mast cells.

Mast cells suspended in PBS containing 5.4 mM glucose (10^7 to 3×10^7 cells/1.5 ml) were incubated with [8-^{14}C] adenine (2.5 μCi, 42 nmoles) at 37° for 30 min. The cells were then washed three times with 5 ml PBS containing 0.1 mM nonradioactive adenine. About 1 per cent of the added [8-^{14}C]-adenine was incorporated into mast cells. The labeled cells were resuspended in PBS containing the substances to be tested, and incubated for up to 30 min as above.

The reaction was terminated by immersion of the tubes into boiling water for 3 min. After centrifuging at 500 *g* for 5 min at 0°, the supernatant fluids were mixed with 1 μmole each of nonradioactive adenine, AMP, cyclic AMP, ADP and ATP. [^{14}C] cyclic AMP was separated by the procedure adapted from that of Ramachandran [25] and of Mao and Guidotti [26].

The sample was applied to a column (0.9 × 15 cm) of neutral aluminum oxide previously equilibrated with 10 mM Tris–HCl (pH 7.4), and eluted with 3 ml of the same buffer. The eluate was directly poured onto a second column (0.9 × 15 cm) of Dowex 1 × 8 (Cl^- form). The column was washed once with 20 ml of the same buffer, and the [^{14}C] cyclic AMP was eluted with 3 ml of 0.1 N HCl. Radioactivity of aliquots (1 ml) was counted in 15 ml of NT-scintillant (nonylphenopolyethoxyether, 300 ml; toluene, 700 ml; and 2,5-diphenyloxazole, 4 g) recommended by Kawakami *et al.* [27]. Radioactivity counts were corrected for recovery of unlabeled cyclic AMP (80.2 ± 1.5 per cent) determined by optical density at 260 nm [6].

Mast cell labeled with [8-^{14}C] adenine and [^{32}P] phosphate. A method applied to rat leg muscle cells by Reporter [28] was applied to mast cells.

Mast cells washed three times with Fischer's medium were suspended in the same medium (4×10^5 cells/1 ml), and incubated in the presence of [8-^{14}C] adenine (5 μCi) for 2 hr at 37° and then for another 30 min after the addition of [^{32}P] phosphate (10 μCi). The cells were washed three times with 10 ml PBS containing 0.1 mM unlabeled adenine, and resuspended in 0.5 ml of 5% perchloric acid. After freeze-thawing the cell suspension ten times followed by centrifuging at 500 *g* for 10 min, the supernatant fluid was neutralized with 2 M $KHCO_3$, and mixed with 1 μmole each of unlabeled cyclic AMP and other carriers.

Nucleotides were separated on a column (1 × 8 cm) of Dowex 1 × 8 (200–400 mesh, formate form) by a slight modification of the method of Heidrick and Ryan [29]. The column was eluted successively with 1.5 M formic acid, 4 M formic acid–0.2 M ammonium formate, and 4 M formic acid–1.5 M ammonium formate at the flow rate of 50 ml/hr at room temperature.

Radioactivity of aliquots (1 ml) was counted in 15 ml of NT-scintillant [27]. The counter was adjusted so that 44 per cent of the ^{32}P counts entered the ^{14}C channel, while no ^{14}C counts entered the ^{32}P channel.

Fractions from the column were also monitored at 260 nm, and, if necessary, the nucleotides were identified by thin-layer chromatography of the concentrated aliquots according to Dighe *et al.* [30].

Estimation of ATP. ATP was estimated by the luciferin–luciferase method of Bihler and Jeanrenaud [31].

Sucrose density gradient centrifugation of mast cell granules. Mast cells (3 to 5×10^6 cells) were incubated in 0.5 ml of 0.25 M sucrose–1 mM Tris–HCl (pH 8.0) or the same medium containing 5 mM cyclic AMP for 5 min at 37°, prior to the treatment with compound 48/80 (10 μg/ml) for 30 sec.

The cells were immediately cooled, and subjected to a sucrose density gradient centrifugation according to Martin and Ames [32]. The cooled cells were layered on top of a linear sucrose density gradient (0.25 to 1.4 M, 4 ml) in a Hitachi RPS 40 rotor, and centrifuged at 100,000 *g* for 30 min at 4°. Fractions of six drops were collected, and assayed for histamine [20]. Granules appeared at a sucrose density of about 0.65 M.

Chemicals. Cyclic AMP and dibutyryl cyclic AMP were obtained from Seishin Pharmaceutical Co., Tokyo, Japan. Compound 48/80 was purchased from Burroughs Wellcome & Co., Tuckahoe, N.Y., U.S.A. Prostaglandin E_1 (PGE_1) was a gift from Ono Pharmaceutical Co., Osaka, Japan, and dissolved in ethanol (0.1 ml/mg) and diluted with 0.2% Na_2CO_3 solution (0.9 ml/mg) prior to use. Epinephrine (1 mg of HCl salt/ml) was purchased from Sankyo Pharmaceutical Co., Tokyo, Japan. [8-^{14}C] adenine (59 mCi/m-mole), [^{32}P] phosphate (carrier-free, 1 mCi) and [^{3}H] cyclic AMP (27 Ci/m-mole) were purchased from Amersham–Searle. Other chemicals of reagent grade were obtained commercially.

RESULTS

Effect of epinephrine, theophylline and prostaglandin E_1 on histamine release and cyclic AMP accumulation in mast cells. As shown in Fig. 1A, epinephrine, at concentrations from 6.25×10^{-5} to 5×10^{-4} M,

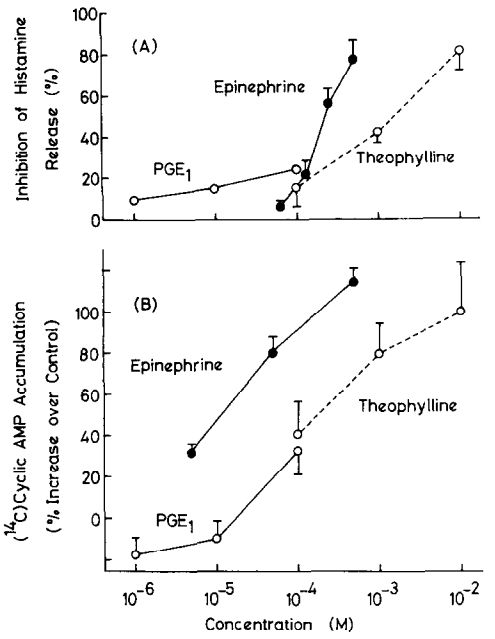

Fig. 1. Effect of epinephrine, theophylline and prostaglandin E_1 on the release of histamine induced by compound 48/80 (A), and on the accumulation of cyclic AMP (B) in mast cells. (A) Rat peritoneal mast cells were preincubated with various concentrations of epinephrine, theophylline or PGE_1 for 15 min at 37° prior to the treatment with compound 48/80 (1 μg/ml) for 5 min. Histamine was assayed as described in Methods and Materials. The total content of histamine was 20.4 μg/10^6 cells. (B) Mast cells preincubated with [8-^{14}C]adenine were incubated with epinephrine, theophylline or PGE_1 as in (A), and the [^{14}C]cyclic AMP formed was separated and counted as described in Methods and Materials. The basal count of [^{14}C] cyclic AMP in control cells was 110.3 ± 6.6 cpm/10^6 cells. Values were the mean of three incubations.

strongly and dose dependently inhibited the release of histamine from isolated mast cells induced by compound 48/80. Epinephrine was also a powerful stimulator, at concentrations from 5×10^{-5} to 5×10^{-4} M, for the accumulation of [^{14}C] cyclic AMP in mast cells previously incubated with [8-^{14}C] adenine (Fig. 1B).

Theophylline was also a potent inhibitor of histamine release and a powerful stimulator for the [^{14}C] cyclic AMP accumulation in mast cells. However, its effective doses were somewhat higher than those of epinephrine.

In contrast, PGE_1 in the concentration range used was a weak inhibitor for histamine release. Furthermore, PGE_1, at the highest concentration (10^{-4} M) used, only slightly (1.3-fold of the basal) stimulated the formation of [^{14}C] cyclic AMP, although, in one experiment, it elevated the cyclic AMP content about 2-fold (from the basal 4.2 pmoles to 9.6 pmoles/10^6 cells).

Time course of effects of epinephrine and theophylline. As shown in Fig. 2A, on exposure to epinephrine, the formation of [^{14}C] cyclic AMP in mast cells previously labeled with [8-^{14}C] adenine rapidly increased, reached the maximal level within 5 min, and then declined slowly to the basal level over the next 25 min. In contrast, the total cellular content of cyclic AMP increased more slowly, reached its peak within 15 min, and decreased gradually thereafter.

Therefore, the specific activity of [^{14}C] in [^{14}C] cyclic AMP rapidly reached a maximum (2.4-fold of the basal) at about 5 min after the cells were exposed to epinephrine, and then fell rapidly thereafter.

The inhibition of histamine release became detectable within 5 min, maximal within 15 min, and remained elevated at 20 min after exposure to epinephrine.

On the other hand, theophylline, an inhibitor of phosphodiesterase activity [33], rapidly elevated the cyclic AMP content to a near maximal level within 10 min of the exposure, and maintained the elevated level thereafter. In contrast, the accumulation of [^{14}C] cyclic AMP and the inhibition of histamine release slowly increased over 30 min of the treatment (Fig. 2B). No significant increase in the specific activity of [^{14}C] in [^{14}C] cyclic AMP was observed during 30 min after exposure to theophylline.

To maintain the elevated level of cyclic AMP, the continued presence of theophylline seemed to be necessary. When mast cells incubated with 10^{-2} M theophylline for 20 min were washed, and reincubated in the fresh PBS medium containing no theophylline, the cellular cyclic AMP level returned to a near baseline level within 10–15 min. Concomitantly, the histamine release which had been maximally inhibited by theophylline was rapidly elevated to the control level (Fig. 3).

Effect of epinephrine on specific activities of ATP and cyclic AMP in double-labeled mast cells. As shown in Table 1, the specific activity of [^{32}P] in cyclic AMP of the control cells was more than 10-fold higher than that in ATP. Accordingly, although mast cells were not completely equilibrated to the [^{14}C] adenine label even after 2.5 hr, the ratio of the specific activities, [^{32}P]/[^{14}C], in cyclic AMP was 6-fold higher than that in ATP.

Epinephrine caused a marked increase in the specific activity of [^{14}C] in cyclic AMP, but caused only a slight change in that in ATP. On the other hand, the specific activity of [^{32}P] in both ATP and cyclic AMP was not altered much by epinephrine. Accordingly, [^{32}P]/[^{14}C] in cyclic AMP in epinephrine-treated cells was reduced to about $\frac{1}{5}$ of that in the control cells. This marked change in [^{32}P]/[^{14}C] probably indicates the presence of a special pool of ATP available for the epinephrine-induced formation of cyclic AMP in mast cells.

Histamine release from mast cells by compound 48/80 in the presence or absence of NaCl. As shown in Fig. 4, the histamine release from mast cells induced by compound 48/80 was very rapid in 0.154 M NaCl, being maximally elevated within 5 min. In contrast, when the cells were treated with this liberator in 0.25 M sucrose in the absence of NaCl, about one-half of the cellular histamine was quickly released within 5 min, and the liberation plateaued thereafter, regardless of whether the incubation was continued in the same medium or in water after the sucrose-treated cells were sedimented at 2700 *g* for 15 min.

However, when the cells incubated in a 0.25 M sucrose medium were exposed to 0.154 M NaCl, a

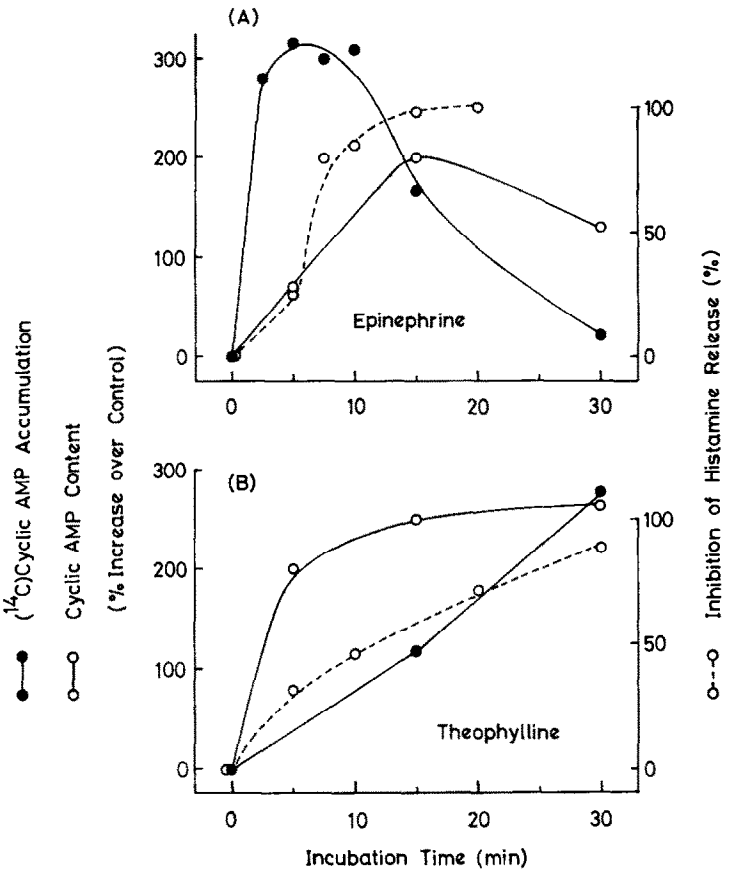

Fig. 2. Effect of epinephrine and theophylline on cyclic AMP accumulation, cyclic AMP content, and histamine release in mast cells. (A) Mast cells or mast cells previously labeled with [8-^{14}C] adenine were incubated in the presence of 5×10^{-4} M epinephrine at 37° for up to 30 min. The [^{14}C] cyclic AMP formed, cyclic AMP content, and histamine released by the addition of compound 48/80 (1 μg/ml) were estimated as described in the text. The count of [^{14}C] cyclic AMP and the content of cyclic AMP (cpm/pmole 10^6 cells) were: 76.3/3.6 at 0-time, 324/6.1 at 5 min, 201/10.8 at 15 min, and 95.8/8.3 at 30 min respectively. (B) Mast cells or mast cells prelabeled with [8-^{14}C] adenine were preincubated in the presence of 1×10^{-2} M theophylline. Other experimental conditions were similar to those in (A). The count of [^{14}C] cyclic AMP and the content of cyclic AMP (cpm/pmole/10^6 cells) were: 72.8/3.8 at 0-time, 159.8/13.3 at 15 min, and 273.7/13.8 at 30 min respectively.

a significant release of the remaining histamine was observed.

The basal leakage of histamine in isotonic NaCl or sucrose solution was negligible.

Effects of cyclic AMP, dibutyryl cyclic AMP, epinephrine and theophylline on step I and step II reactions. Table 2 shows that cyclic AMP and dibutyryl cyclic AMP were effective in inhibiting histamine release from mast cells induced by compound 48/80 in an isotonic sucrose solution (step I), but less effec-

Table 1. Effect of epinephrine on specific activities of ATP and cyclic AMP in double-labeled mast cells*

	Nucleotide	Content (pmoles/10^6 cells)	[^{14}C] (cpm/pmole)	[^{32}P]	[^{32}P]/[^{14}C]
Control	ATP	457 ± 42	2.2	0.1	0.05
	Cyclic AMP	3.0 ± 0.2	5.4	1.5	0.28
Epinephrine	ATP	337 ± 17	1.9	0.2	0.11
	Cyclic AMP	6.4 ± 0.2	17.4	0.9	0.05

* Mast cells suspended in Fischer's medium (4×10^5 cells/1.0 ml) were incubated with [8-^{14}C] adenine (5 μCi) for 2 hr at 37°, and for another 30 min after the addition of [^{32}P] phosphate (10 μCi). Epinephrine (5×10^{-4} M) was present during the last 10 min of labeling with [^{32}P] phosphate. Nucelotides were extracted with 5% perchloric acid and separated on a Dowex 1 × 8 (formate form) column as described in the text [29].

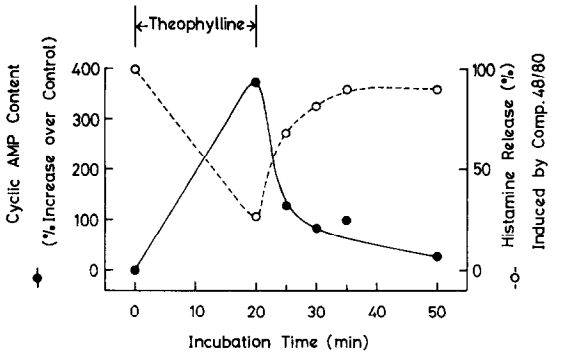

Fig. 3. Effect of theophylline on cyclic AMP content and histamine release in mast cells. Rat peritoneal mast cells were incubated at 37° for 20 min in the presence of 1×10^{-2} M theophylline. Then the cells were washed three times with PBS and incubated in the fresh medium without theophylline. At intervals, an aliquot of the incubation mixture was taken, and assayed for cyclic AMP content and for histamine released by adding compound 48/80 (1 μg/ml) as described in the text. The basal content of cyclic AMP was 4.2 pmoles/10^6 cells. Compound 48/80 released 80.5 ± 3.5 per cent (taken as 100) of the total histamine (18.5 ± 2.1 μg/10^6 cells) from the intact cells.

tive in suppressing the subsequent amine release from the treated cells in the presence of NaCl (step II).

Theophylline and epinephrine were also effective in suppressing the histamine release in step I, but almost ineffective in inhibiting the amine release in step II.

Also in isolated granules, cyclic AMP and dibutyryl cyclic AMP did not significantly affect the basal histamine release in water, and did not suppress the histamine release which had been maximally elevated by 0.154 M NaCl (Table 3).

Mast cells, which had been preincubated with cyclic AMP and then treated with compound 48/80 in an isotonic sucrose solution, were also examined by a sucrose density gradient centrifugation; the results indicated that cyclic AMP suppressed the extrusion of granules from the cells (Fig. 5).

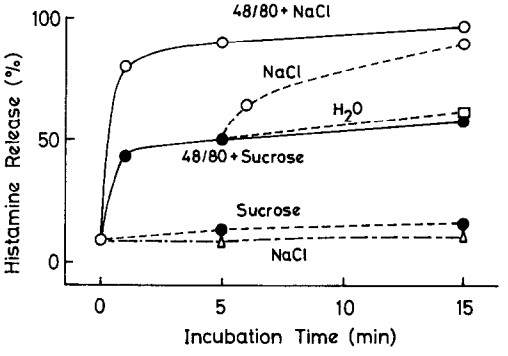

Fig. 4. Effect of NaCl on histamine release in mast cells. Rat peritoneal mast cells were incubated at 37° in 0.154 M NaCl containing compound 48/80 (1 μg/ml) (○——○); 0.25 M sucrose containing compound 48/80 (●——●); 0.154 M NaCl (Δ- · -Δ); or 0.25 M sucrose (●---●). After 5 min, aliquots of mast cells incubated in 0.25 M sucrose containing compound 48/80 were sedimented at 2700 *g* for 15 min, washed with water, and incubated for another 10 min in 0.154 M NaCl (○---○) or in water (□---□). The basal level of histamine was 14.8 ± 1.4 μg/10^6 cells. Values were the mean of duplicate samples.

Table 2. Effects of cyclic AMP, dibutyryl AMP, epinephrine and theophylline on step I and step II reactions*

Compound	Concn (mM)	Inhibition of histamine release (%) Step I	Step II
Cyclic AMP	5.0	57.4 ± 8.8	29.5 ± 3.7
Dibutyryl cyclic AMP	5.0	35.4 ± 7.4	12.2 ± 6.3
Epinephrine	0.5	22.1 ± 3.5	0
Theophylline	10.0	48.8 ± 3.0	0

* Step I: Mast cells (10^5 cells/tube) were incubated in 1 ml of 0.25 M sucrose–1 mM Tris–HCl (pH 7.2) containing various agents for 15 min at 37° prior to the treatment with compound 48/80 (10 μg/ml) for 5 min. After centrifuging the incubation mixture at 2700 *g* for 15 min, histamine in the supernatant fluid was assayed as described in the text. The per cent release of the total histamine (14.2 ± 3.3 μg/10^6 cells) by compound 48/80 alone in the control experiment was 48.1 ± 2.0. Step II: After the step I reaction, cells and granules sedimented at 2700 *g* were washed once with distilled water, resuspended in 0.154 M NaCl containing various agents, and incubated for 10 min at 37°. Histamine in the supernatant fluid was assayed as above. The amount of histamine retained in the 2700 *g* pellet after step I was 7.1 ± 1.9 μg/10^6 of the original cells (= 50.2 per cent recovery). The per cent release of the remaining histamine by 0.154 M NaCl in the control experiment was 98.2 ± 3.8. Values were the mean ± S.E.M. of three to four incubations.

DISCUSSION

Our present experiments indicate that cyclic AMP plays an important role in mediating the inhibitory effect of epinephrine and theophylline on histamine release in mast cells.

Table 3. Effects of cyclic AMP and dibutyryl cyclic AMP on histamine release from isolated granules of mast cells*

Compound	Concn (mM)	Per cent release of histamine
None (distilled water)		33.1 ± 3.3
Compound 48/80	(1 μg/ml)	39.2 ± 5.2
Cyclic AMP	5.0	40.2 ± 2.3
Dibutyryl cyclic AMP	5.0	41.0 ± 1.3
NaCl	1.0	40.2 ± 4.0
	10.0	71.8 ± 1.0
	100.0	96.8 ± 5.1
NaCl + cyclic AMP	100.0 + 5.0	90.3 ± 1.3
NaCl + dibutyryl cyclic AMP	100.0 + 5.0	90.6 ± 3.5

* Granules were isolated from rat peritoneal mast cells (5×10^6 cells containing 81 μg histamine) as described in the text. Granules (65.6 μg histamine, 81 per cent recovery) were washed three times with deionized water, and resuspended in water. Aliquots (1 ml, 2.2 μg histamine) of the granule suspension were incubated with or without the nucleotides for 5 min at 0° prior to the treatment with NaCl for 10 min. After centrifuging the incubation mixture at 2700 *g* for 15 min, histamine in the supernatant fluid was assayed as above. Values were the mean ± S.E.M. of three incubations.

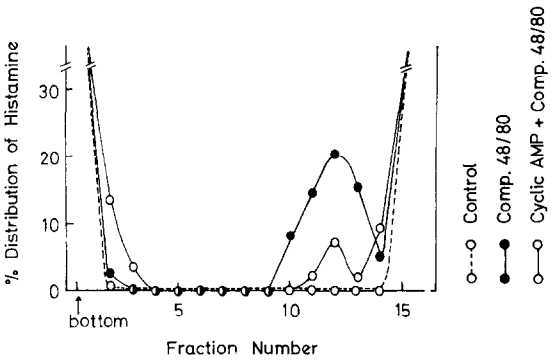

Fig. 5. Sucrose density gradient centrifugation of mast cells treated with compound 48/80 in the presence or absence of cyclic AMP. Mast cells (3.7×10^6 cells, 50 μg of the total histamine) suspended in 0.5 ml of 0.25 M sucrose–1 mM Tris-HCl (pH 8.0) (○---○), or in the vehicle containing 5 mM cyclic AMP were incubated at 37° for 5 min, and then treated with compound 48/80 (10 μg/ml) for 30 sec (○——○ and ●——●). The mixture was subjected to a linear sucrose density gradient (0.25 M to 1.4 M, 4 ml) centrifugation (100,000 *g* for 30 min, 4°). Fractions of six drops were collected, and the histamine content was determined as described in the text. The per cent distribution of histamine relative to the base line values was plotted.

Both epinephrine and theophylline, at concentrations which were effective in suppressing the histamine release from mast cells induced by compound 48/80, stimulated the formation of [^{14}C] cyclic AMP in the cells previously labeled with [8-^{14}C] adenine (Fig. 1).

In contrast, PGE_1, which was reported to inhibit compound 48/80-induced [3] and the immunological [4] histamine release from mast cells, was not very effective in suppressing histamine release at 10^{-4} M under the experimental conditions used, although it slightly stimulated the formation of [^{14}C] cyclic AMP in the prelabeled cells and also elevated the cyclic AMP content.

Our results with PGE_1 were similar to those reported by Johnson *et al.* [34]. They found that PGE_1 (10^{-4} M) alone or in combination with papaverine (10^{-2} M) did not affect the response of mast cells to compound 48/80, and also that PGE_1 alone was not effective in elevating cyclic AMP content, but was effective in the presence of papaverine.

The reason for these differences in results is not clear yet. As Johnson *et al.* [34] suggested, it might be due to the difference in the degree of homogeneity of mast cell preparations used [3], or due to the difference in the mechanism of histamine release induced by different liberators.

Although both epinephrine and theophylline were potent inhibitors for histamine release, time courses of their effects were different (Fig. 2).

Before the inhibitory effect of epinephrine on histamine release became detectable, it had induced a rapid formation of [^{14}C] cyclic AMP with a high specific radioactivity in mast cells previously incubated with [8-^{14}C] adenine within 5 min of the introduction of the drug.

On the other hand, theophylline rapidly elevated the cellular cyclic AMP content, while it only slowly increased the radioactive nucleotide and gradually suppressed histamine release. In contrast to epinephrine, theophylline did not significantly elevate the specific activity of [^{14}C] cyclic AMP during a 30-min incubation.

This difference in time course for the accumulation of total and radioactive cyclic AMP in response to epinephrine and theophylline could indicate that the formation of cyclic AMP from the special pool(s) of ATP, but not the elevation of total cyclic AMP content, is required for the suppression of histamine release in mast cells.

These results were similar to those reported by Krishna *et al.* [35] for the effect of norepinephrine on both total and radioactive cyclic AMP in [^{3}H] adenine-labeled slices of rat cerebral cortex, but in contrast to those reported by Schultz and Daly [36] in similarly labeled guinea pig cerebral cortex in response to histamine. Therefore, the presence of a special intracellular pool(s) of ATP for the formation of cyclic AMP needs further investigation as to the sources of tissues or cells and reagents used.

In this regard, the presence of more than one pool of ATP available for the formation of cyclic AMP in mast cells was indicated in our studies with the cells which had been double-labeled with [8-^{14}C]-adenine and [^{32}P] phosphate (Table 1). In nonstimulated control cells, the ratio of the specific activities, [^{32}P]/[^{14}C], in cyclic AMP was unexpectedly (6-fold) higher than that in ATP. On exposure of mast cells to epinephrine for the last 10 min of [^{32}P]-labeling, [^{32}P]/[^{14}C] in cyclic AMP was reduced to one-half of that in ATP due to the marked increase in [^{14}C] cyclic AMP formation with a less significant alteration in [^{32}P] cyclic AMP formation.

According to Uvnäs *et al.* [11–14], a simple model for the storage and release of biogenic amines in mast cells is as follows: (1) mast cell granules have the property of a weak anion exchanger; (2) both histamine and serotonin are bound to the carboxyl groups of the heparin–protein complex, but not directly to the granular heparin (Lagunoff [37] has suggested the partial participation of anionic sites of heparin for binding the amines and cations); and (3) the amine release induced by compound 48/80 [38] or antigen [39] can be explained by an ionic exchange between the granular amines and the extracellular cations (step II) after the primary exposure of granules to the external environment, either by degranulation, or through the newly formed intracellular cavities which communicate with the external space (step I).

Our results, obtained with isolated granules (Tables 2 and 3) and sucrose density gradient centrifugation of mast cells treated with compound 48/80, in the absence or presence of cyclic AMP (Fig. 5), indicated that cyclic AMP inhibits histamine release from mast cells mainly by interfering with the primary degranulation step (step I), but not with the ionic exchange step (step II).

A similar inhibitory function of the intracellular cyclic AMP on an early stage of histamine release was also suggested by Bourne *et al.* [7] with leukocytes of allergic donors. According to Lichtenstein [40], the antigen-induced, IgE-mediated histamine release from human leukocytes can be separated into two stages: a primary antigen-dependent, Ca^{2+}-independent stage, and a secondary antigen-independent,

Ca^{2+}-dependent stage. Dibutyryl cyclic AMP, methylxanthines and catecholamines have been shown to inhibit histamine release in the first stage (antigen contact with cells), but have little inhibitory effect on the second stage [7].

Step I appears to be a complicated, sequential reaction. On exposure to compound 48/80 [38] or antigen [39], mast cells were swollen followed by fusion of perigranular and cellular membranes, and by extrusion of granules or by the formation of intracellular cavities to expose granules to the outer space.

Furthermore, the histamine-releasing mechanism of compound 48/80 also seemed to be complicated. Gillespie [41] reported a rapid decrease in the formation of [^{3}H] cyclic AMP in rat peritoneal mast cells which had been prelabeled with [^{3}H] adenine. Sullivan and Parker [42] observed an activation of a high K_m cyclic AMP phosphodiesterase activity of rat mast cells by compound 48/80. In contrast, according to Johnson *et al.* [34], compound 48/80, at a concentration of 2 μM [= 1 μg/ml, using the molecular weight of the trimer (520)] that released more than 50 per cent of the total histamine, did not affect levels of cyclic AMP.

Therefore, the precise manner in which cyclic AMP affects these sequential reactions awaits further examinations.

REFERENCES

1. A. Ichikawa, H. Hayashi, M. Minami and K. Tomita, *Biochem. Pharmac.* **21**, 317 (1972).
2. A. R. Johnson and N. C. Moran, *J. Pharmac. exp. Ther.* **175**, 632 (1970).
3. L. J. Loeffler, W. Lovenberg and A. Sjoerdsma, *Biochem. Pharmac.* **20**, 2287 (1971).
4. M. Kaliner and K. F. Austen, *J. Immun.* **112**, 664 (1974).
5. L. M. Lichtenstein and S. Margolis, *Science, N.Y.* **161**, 902 (1968).
6. L. M. Lichtenstein and R. DeBernardo, *J. Immun.* **107**, 1131 (1971).
7. H. R. Bourne, L. M. Lichtenstein and K. L. Melmon, *J. Immun.* **108**, 695 (1972).
8. E. S. K. Assem and H. O. Schild, *Nature, Lond.* **224**, 1028 (1969).
9. R. P. Orange, M. A. Kaliner, P. J. Laraia and K. F. Austen, *Fedn Proc.* **30**, 1725 (1971).
10. R. P. Orange, W. G. Austen and K. F. Austen, *J. exp. Med.* **134**, 1365 (1971).
11. I. L. Thon and B. Uvnäs, *Acta physiol. scand.* **71**, 303 (1967).
12. C.-H. Åborg, J. Novotny and B. Uvnäs, *Acta physiol. scand.* **69**, 276 (1967).
13. B. Uvnäs, *Fedn Proc.* **33**, 2172 (1974).
14. B. Uvnäs, *Life Sci.* **14**, 2355 (1974).
15. N. Chakravarty and E. Zeuthen, *J. Cell Biol.* **25**, 113 (1965).
16. R. Dulbecco and M. Vogt, *J. exp. Med.* **99**, 167 (1954).
17. R. E. Bray and P. P. VanArsdel, Jr., *Proc. Soc. exp. Biol. Med.* **106**, 255 (1961).
18. J. P. Kaltenbach, M. H. Kaltenbach and W. B. Lyons, *Expl. Cell Res.* **15**, 112 (1958).
19. B. Uvnäs, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. XXXI(A), p. 395. Academic Press, New York (1974).
20. P. A. Shore, A. Burkhalter and V. H. Cohn, *J. Pharmac. exp. Ther.* **127**, 182 (1959).
21. A. G. Gilman, *Proc. natn. Acad. Sci. U.S.A.* **67**, 305 (1970).
22. E. Miyamoto, J. F. Kuo and P. Greengard, *J. biol. Chem.* **244**, 6395 (1969).
23. J. F. Kuo and E. C. De Renzo, *J. biol. Chem.* **244**, 2252 (1969).
24. H. R. Bourne, R. I. Lehrer, M. J. Cline and K. L. Melmon, *J. clin. Invest.* **50**, 920 (1971).
25. J. Ramachandran, *Analyt. Biochem.* **43**, 227 (1971).
26. C. C. Mao and A. Guidotti, *Analyt. Biochem.* **59**, 63 (1974).
27. M. Kawakami, R. Majima and K. Shimura, *Seikagaku* **44**, 273 (1971).
28. M. Reporter, *Biochem. biophys. Res. Commun.* **48**, 598 (1972).
29. M. L. Heidrick and W. L. Ryan, *Biochim. biophys. Acta* **237**, 301 (1971).
30. P. K. Dighe, D. N. Pahuja and D. H. Shah, *J. Chromat.* **40**, 449 (1969).
31. I. Bihler and B. Jeanrenaud, *Biochim. biophys. Acta* **202**, 496 (1970).
32. R. G. Martin and B. N. Ames, *J. biol. Chem.* **236**, 1372 (1961).
33. R. W. Butcher and E. W. Sutherland, *J. biol. Chem.* **237**, 1244 (1962).
34. A. R. Johnson, N. C. Moran and S. E. Mayer, *J. Immun.* **112**, 511 (1974).
35. G. Krishna, J. Forn, K. Voigt, M. Paul and G. L. Gessa, in *Advances in Biochemical Psychopharmacology* (Eds. P. Greengard and E. Costa), Vol. 3, p. 155. Raven Press, New York (1970).
36. J. Schultz and J. W. Daly, *J. biol. Chem.* **248**, 843 (1973).
37. D. Lagunoff, *Biochemistry* **13**, 3982 (1974).
38. P. Röhlich, P. Anderson and B. Uvnäs, *J. Cell Biol.* **51**, 465 (1971).
39. P. Anderson, S. A. Slorach and B. Uvnäs, *Acta physiol. scand.* **88**, 359 (1973).
40. L. M. Lichtenstein, *J. Immun.* **107**, 1122 (1971).
41. E. Gillespie, *Experientia* **29**, 447 (1973).
42. T. J. Sullivan and C. W. Parker, *Biochem. biophys. Res. Commun.* **55**, 1334 (1973).