Biochemical Pharmacology, Vol. 21, pp. 317–331. Pergamon Press, 1972. Printed in Great Britain

AN ACUTE INFLAMMATION INDUCED BY INORGANIC PYROPHOSPHATE AND ADENOSINE TRIPHOSPHATE, AND ITS INHIBITION BY CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE

ATSUSHI ICHIKAWA, HIDEYA HAYASHI, MACHIKO MINAMI and KENKICHI TOMITA

Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

(*Received* 8 *September* 1970; *accepted* 16 *July* 1971)

Abstract—A subcutaneous injection of inorganic pyrophosphate (PP_i) produced characteristic inflammatory lesions on rat skins, and the rats displayed acute pain reactions at the time of administration. The increase in vascular permeability due to this PP_i-induced inflammation was immediate and monophasic with no delayed response. Furthermore, this vascular response was synchronized with an increase in cutaneous histamine, indicating that PP_i-induced inflammation was mediated by endogenous histamine. ATP showed similar and more potent effects than PP_i on changes in vascular permeability and cutaneous histamine without causing acute pain reactions. Both the vascular response and histamine release elicited by PP_i were inhibited by simultaneous administration of epinephrine, methylxanthines and by cyclic 3′,5′-adenosine monophosphate (cAMP), but not by any other adenine nucleotide or cyclic nucleoside monophosphate, except for 5′-AMP, which inhibited the increase in cutaneous histamine. cAMP was also effective in inhibiting both effects induced by ATP, while 5′-AMP suppressed only the increase in cutaneous histamine induced by ATP. PP_i released histamine from isolated mast cells, but did not significantly release it from leukocytes. The release of histamine from mast cells induced by PP_i as well as by ATP and compound 48/80 was also inhibited by cAMP and 5′-AMP.

DURING studies on specific chemical reactions of thyroid hormones with various phosphate derivatives, Tomita *et al.*[1] observed that rats showed acute pain reactions (screaming, body writhing and biting) when they received subcutaneous injections of a reaction mixture of 3,5,3′,5′-tetraiodothyroacetic acid with inorganic pyrophosphate (PP_i) or a solution of PP_i alone.[1]

We confirmed these findings and further observed that injection of a PP_i solution produced characteristic inflammatory lesions on rat skins. The instant PP_i was injected, rats showed severe pain reactions and, in a few hours, local swelling and erythema were observed at the site of injection. When daily injections of PP_i were continued for about 2 weeks, the skin lesion became covered with a scab and exfoliation sometimes ensued.

This led us to investigate the inflammatory process induced by PP_i. We observed that the injection of PP_i caused immediate and temporary increases in local vascular permeability and cutaneous histamine content, and that both responses to PP_i were suppressed by simultaneous administration not only of epinephrine, which is a local anti-inflammatory agent[2,3] and a stimulator of cyclic 3′,5′-adenosine monophosphate (cAMP) formation in various tissues,[4] but also of theophylline, an inhibitor for cAMP phosphodiesterase (EC 3.1.4c).[5] Lichtenstein and Margolis[6]

reported similar inhibitions of allergic histamine release from human leukocytes by catecholamines and methylxanthines, and suggested a possible regulatory role for cAMP in this process.

In fact, simultaneous administration of cAMP very effectively suppressed inflammatory responses of rat skins to PP_i. In contrast, ATP augmented PP_i-induced inflammation and ATP alone also elicited inflammatory effects similar to those of PP_i on rat skins. This ATP-induced inflammation was also inhibited by cAMP.

We also observed that PP_i caused histamine release from isolated mast cells of rats in a manner similar to that of ATP, as reported by Diamant and Krüger,[7] and that this histamine release *in vitro* by both PP_i and ATP was effectively suppressed by cAMP.

In the present report, effects of PP_i on vascular permeability, cutaneous histamine, and histamine release from mast cells are compared with those of ATP, especially with respect to the inhibitory influence of cAMP.

METHODS AND MATERIALS

Adult Wistar rats of both sexes weighing 150–200 g were used and fed a standard rat chow (NIHON CLEA, CE-2 pellet) and water *ad lib.*

Vascular permeability test. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and 10 min later received 1% Evans blue solution (2 ml/kg) through the tail vein. After another 10 min, various amounts of PP_i or other compounds dissolved in 0·025–0·05 ml of 0·9% saline (pH 7·2–7·4) were administered at selected sites on the ventral skin, which had been closely clipped (one injection/ca. 2×2 cm^2, 4–6 sites/rat). Control rats were injected with 0·05 ml of 0·9% saline. At selected intervals, animals were decapitated and skin fragments (ca. 200 mg) excised from injected areas were minced, digested and extracted for the dye by the method of Beach and Steinetz.[8] Evans blue extracted in chloroform was measured at 620 nm.

Estimation of histamine in rat skin. Skin fragments (ca. 200 mg) prepared as above, omitting the Evans blue injection, were homogenized with 5 ml of 0·4 perchloric acid. Histamine was extracted from the homogenate and assayed fluorometrically by the method of Shore *et al.*[9]

Isolation of leukocytes and mast cells. From heparinized rat blood (heparin, 100 units i.v./rat) leukocytes were isolated by a slight modification of the method of Lichtenstein and Osler.[10] The leukocyte-rich fraction, which sedimented at 100 *g* for 8 min, was washed three times with a buffered medium containing 154 mM NaCl, 2·7 mM KCl, 0·9 mM $CaCl_2$, 6·7 mM phosphate buffer (pH 7·0) and bovine serum albumin (BSA) (1 mg/ml). At this stage, the ratio of leukocytes to erythrocytes was about 4 : 1.

Leukocytes were also obtained, using the method of Stähelin *et al.*[11] from the peritoneal exudate of rats which had been intraperitoneally injected with 20 ml of 0·9% saline 18 and 1 hr before sacrifice. These were washed with the same medium as before.

Mast cells were isolated from the peritoneal cavity fluid by a slight modification of the method of Chakravarty and Zeuthen.[12] The suspension medium I[12] was replaced with the medium used above for leukocytes. The cell suspension was layered on a 30% BSA solution instead of on two layers of 30 and 40% BSA solutions. It

was then centrifuged at 220 *g* for 5 min at 0–4°. The bottom fraction containing mast cells was washed three times with the same medium.

Leukocytes and mast cells were finally suspended in more of the medium to a level of about 5×10^4 to 10^5 cells per ml and were immediately used.

Histamine assay in studies in vitro. Leukocytes or mast cells (ca. 10^5 cells) suspended in 1 ml of the medium, containing various required reagents, were incubated in polyethylene tubes (15 × 100 mm) at 37° for 30 min in most cases. The reaction was terminated by cooling the mixture to ice-cold. After centrifugation at 1000 *g* for 5 min, histamine in the supernatant fraction was assayed by the method of Shore *et al.*[9] The total content of histamine in intact cells was the amount of histamine extracted from cells which had been frozen and thawed five times.

cAMP was obtained from Seishin Pharmaceutical Company, Tokyo, Japan. Compound 48/80 was purchased from Burroughs Wellcome & Company, Tuckahoe, N.Y. Bradykinin was a kind of gift from Dr. T. Suzuki of the Protein Research Institute, Osaka University, Osaka, Japan. Other chemicals of reagent grade and drugs were obtained commercially.

RESULTS

Effect of PP_i on vascular permeability and cutaneous histamine content. Exudation of local blue dye, which was nearly maximal within 30 min, was observed after a subcutaneous injection of PP_i (2·5 μmoles), but not after injecting an equivalent amount of inorganic orthophosphate (P_i). Of the other phosphate derivatives tested, ATP was as effective as PP_i in causing skin bluing, but 5′-AMP and cAMP were almost without effect (Fig. 1).

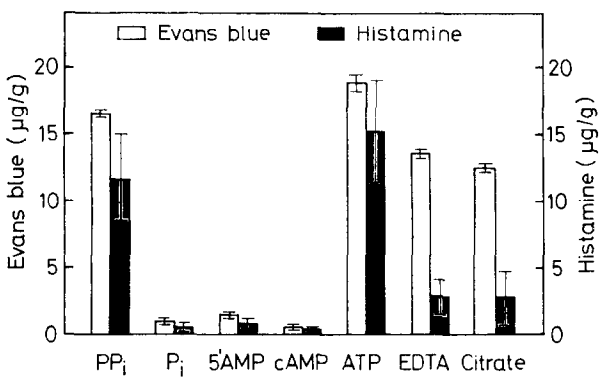

FIG. 1. Effect of PP_i and various other compounds on vascular permeability and cutaneous histamine content in rats. Two groups of six rats, one group pre-injected with Evans blue, were subcutaneously injected at selected sites of the ventral skin either with 2·5 μmoles each of the compounds to be tested, which were dissolved in 0·9% saline, or with saline as the control. After 30 min, the rats were decapitated and fragments of skin were excised from injected areas. Vascular permeability and cutaneous histamine were assayed as described under Methods and Materials. The vertical bars represent the mean (± S.E.) of the values corrected for the saline control values.

Similar vascular responses were also observed with injections of complexing agents like EDTA and citrate. However, as shown in Fig 1, the cutaneous histamine content was markedly increased along with the elevation of vascular permeability by PP_i and

was more markedly increased by ATP, but only slightly increased by EDTA and citrate. P_i, 5'-AMP or cAMP did not significantly affect the cutaneous histamine content.

Although similarities between the inflammatory effects of PP_i and ATP were observed, the animals exhibited apparent acute pain reactions, which were often accompanied by characteristic local muscle trembling only after injection with PP_i. In addition, when the injections (50 μmoles, twice daily) were continued, progressive tissue damage at the sites of injection (including scab formation and its exfoliation) was observed with the administration of PP_i, but not with ATP, EDTA, citrate or P_i.

Though rats showed acute pain reactions at the time of PP_i injection, these reactions did not seem to be directly related to the elevated secretion of catecholamines. Catecholamine contents of the blood and adrenals, as determined by the method of von Euler and Floding,[13] were not altered during the first 60 min after PP_i injection. (In blood: 0·9% saline, 0·05 ml injected, catecholamines 0·013 μg/ml; PP_i 5 μmoles injected, 0·008 μg/ml. In adrenals: saline controls, 0·16 μg/g of tissue; PP_i injected, 0·15 μg/g. The mean of six samples.)

Furthermore, even with prolonged administration of PP_i (50 μmoles, twice daily), no significant change in blood glucose as estimated by Somogyi's method[14] was observed (0·9% saline, 0·05 ml injected, controls: 0 time, 118 $\pm$ 9 mg/100 ml; 20 min, 110 $\pm$ 5; 1 day, 95 $\pm$ 9; 2 days, 105 $\pm$ 6; 6 days, 105 $\pm$ 7; 15 days, 105 $\pm$ 8. PP_i injected: 0 time, 104 $\pm$ 5 mg/100 ml; 20 min, 118 $\pm$ 6; 1 day, 104 $\pm$ 10; 2 days, 108 $\pm$ 3; 6 days, 101 $\pm$ 4; 15 days, 115 $\pm$ 7.)

Time course of changes in vascular permeability and cutaneous histamine content by PP_i. Figure 2 shows that the intensity of the skin's blue color increased to a maximum within 30 min after PP_i injection in rats, and that both its peak and duration correlated well with those of the increase in cutaneous histamine.

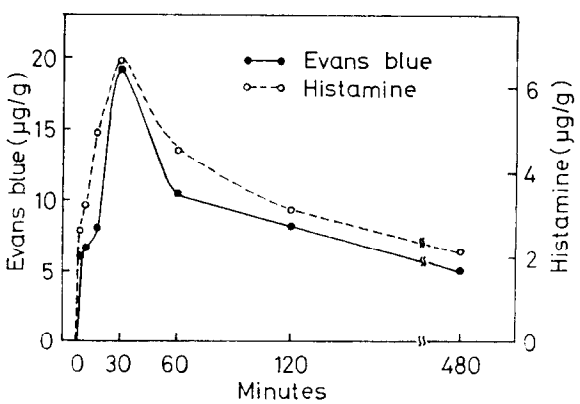

FIG. 2. Time course of the changes in vascular permeability and cutaneous histamine content induced by PP_i. Rats were subcutaneously injected with 0·5 ml of 50 mM PP_i at 0 time. At various intervals, animals were decapitated and vascular permeability and cutaneous histamine content were assayed as described in the text.

The vascular permeability response to PP_i was immediate and monophasic, not biphasic as in thermal injury[15-17] or with other phlogistins, i.e. the Arthus reaction,[15] acute bacterial infection,[18] croton oil[19] and turpentine.[20] No delayed second peak appeared, even after 8 hr.

ATP also induced similar monophasic changes in vascular permeability and in histamine contents of rat skins with a maximum about 30 min after ATP injection. Furthermore, the monophasic permeability response to PP_i was observed not only with rats, but also with guinea-pigs as well.

Effect of an antihistamine and compounds 48/80 *on PP_i-induced and ATP-induced changes in vascular permeability.* Both PP_i-induced, and ATP-induced changes in vascular permeability were strongly inhibited when PP_i or ATP was injected together with diphenhydramine. About 80–90 per cent of the effect of 0·5 μmole of PP_i and ATP was suppressed by 1·25 μmoles of this antihistamine. This dose also inhibited about 90 per cent of the increase in vascular permeability induced by 0·5 μmole of exogenous histamine, but inhibited only 25 per cent of the effect caused by the same amount of serotonin.

Furthermore, the enhanced vascular permeability induced by both PP_i and ATP was strongly suppressed when these compounds were injected in rats which had been depleted of endogenous histamine by preinjection of compound 48/80 [20–22] (Table 1)

TABLE 1. EFFECT OF COMPOUND 48/80 ON CHANGES IN VASCULAR PERMEABILITY INDUCED BY PP_i AND ATP*

	Control	Compound 48/80 treated	Inhibition (%)
	Cutaneous histamine (μg/g)		
0·9% Saline (0·5 ml)	19·5 ± 1·1	4·2 ± 0·6	78
	Evans blue (μg/g)		
PP_i (0·25 μmole)	18·8 ± 2·0	5·0 ± 0·3	73
PP_i (0·025 μmole)	13·0 ± 1·2	3·3 ± 0·3	74
ATP (0·25 μmole)	23·6 ± 3·8	1·8 ± 0·4	92

* Groups of five rats were used. All rats treated with compound 48/80 were given three intraperitoneal injections of this compound at 24-hr intervals (200 μg each twice and 500 μg once). Three hr after the last injection, the rats were subcutaneously injected with PP_i or ATP and killed 30 min later for the assays. Changes in vascular permeability were determined as described in Methods and Materials.

Inhibition of the effect of PP_i by cAMP. In our search for inhibitors of the inflammation elicited by PP_i, we observed potent suppressive effects by epinephrine, methylxanthines and cAMP. As shown in Table 2, about 70 per cent of the effect of 0·5 μmole PP_i on the vascular permeability was abolished when 0·5 μmole cAMP was injected at the same time as PP_i. Epinephrine was as potent as cAMP in inhibiting the effect of PP_i, while theophylline and caffeine were about one-half to two-thirds as effective as cAMP. These inhibitors also suppressed the increase in cutaneous histamine induced by PP_i but generally they were more inhibitory to the increase in vascular permeability than to the increase in cutaneous histamine under the experimental conditions used.

We observed that cAMP was as effective as $N^6,O^{2'}$-dibutyryl cAMP, which has a greater resistance to breakdown by cAMP phosphodiesterase and possibly has a higher penetration rate through cell membranes than cAMP.[4] Suppressive effects

TABLE 2. EFFECTS OF EPINEPHRINE, cAMP AND METHYLXANTHINES ON THE PP_i-INDUCED INCREASES IN VASCULAR PERMEABILITY AND CUTANEOUS HISTAMINE CONTENT*

Compounds injected with PP_i	Doses (μmoles)	No. of samples	Per cent of control	
			Vascular permeability increase	Cutaneous histamine
Epinephrine	0·005	5	35·5 ± 3·5	59·0 ± 1·8
	0·05	5	21·7 ± 3·6	46·6 ± 0·3
	0·5	5	14·8 ± 4·4	35·6 ± 1·1
cAMP	0·005	9	49·7 ± 6·9	69·5 ± 2·9
	0·05	9	32·3 ± 7·1	51·5 ± 1·0
	0·5	9	27·7 ± 5·2	42·5 ± 1·5
Theophylline	0·005	5	67·6 ± 9·2	88·4 ± 1·8
	0·05	5	48·6 ± 3·1	64·5 ± 4·4
	0·5	5	48·6 ± 6·3	53·8 ± 2·1
Caffeine	0·005	5	54·5 ± 6·6	93·4 ± 2·1
	0·05	5	50·4 ± 7·6	78·2 ± 3·5
	0·5	5	44·4 ± 9·2	69·0 ± 1·2

* Rats were subcutaneously injected with 0·025 ml of 20 mM PP_i together with 0·025 ml of one of the inhibitors at various doses, and killed 30 min later for the assays. Control rats were treated with 0·05 ml of 10 mM PP_i. Control values were 21 μg Evans blue/g and 10 μg histamine/g of skin wet weight.

of cAMP on both PP_i-induced changes in vascular permeability and cutaneous histamine were slightly augmented by the presence of methylxanthines ($P < 0·05$; Fig. 3). For these reasons, cAMP was used in all subsequent studies.

cAMP not only suppressed the increase in cutaneous histamine due to the acute inflammation elicited by PP_i, but also fairly well suppressed the vascular response to exogenous histamine. It had a minor influence on the response to serotonin as well (Table 3).

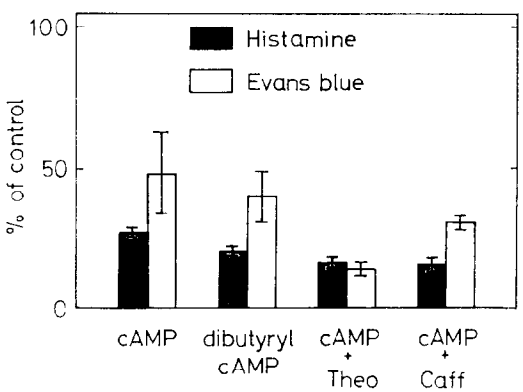

FIG. 3. Effect of cAMP on PP_i-induced inflammation. Groups of five rats were injected with 0·5 μmole of PP_i with or without cAMP or dibutyryl cAMP (0·25 μmole each) or with cAMP and methylxanthines (Theo = theophylline, Caff = caffeine, 0·5 μmole each). After 30 min, vascular permeability and cutaneous histamine were assayed as described in the text.

TABLE 3. EFFECTS OF EPINEPHRINE, cAMP AND METHYLXANTHINES ON THE INCREASED VASCULAR PERMEABILITY INDUCED BY EXOGENOUS HISTAMINE AND SEROTONIN*

Inhibitors (0·25 μmole)	Histamine (0·25 μmole)	Serotonin (0·25 μmole)
	Evans blue (μg/g ± S.E.)	
None (control)	31·2 ± 2·1	16·0 ± 1·3
Epinephrine	7·6 ± 0·2 (76)	5·6 ± 0·1 (65)
cAMP	15·6 ± 0·5 (50)	10·2 ± 1·6 (36)
Theophylline	20·9 ± 3·4 (33)	11·8 ± 1·8 (26)
Caffeine	22·9 ± 3·3 (27)	12·1 ± 2·4 (25)

* Groups of five rats prepared for the vascular permeability test were subcutaneously injected with 0·25 μmole each of histamine or serotonin in combination with 0·25 μmole each of the inhibitors. After 30 min, the rats were decapitated, and Evans blue, extracted from the cutaneous fragments excised from injected areas, was assayed as described in Methods and Materials. The per cent inhibition is shown in parentheses.

In contrast, as shown in Table 4, cAMP at the level of 0·25 μmole only slightly suppressed the increase in vascular permeability induced by various doses of bradykinin, while it inhibited nearly 90 per cent of the increase in cutaneous histamine caused by 1 μg bradykinin. Since cAMP (0·5 μmole) suppressed the effects of PP_i (0·5 μmole) on vascular permeability more effectively than on cutaneous histamine (Table 2), the inflammation induced by PP_i apparently was not directly mediated by the elevated secretion of bradykinin. However, this point should await further investigation, since it is still possible that the PP_i-induced inflammation with the acute pain reactions is mediated by multiple factors including bradykinin.

TABLE 4. EFFECT OF cAMP IN INCREASES IN VASCULAR PERMEABILITY AND CUTANEOUS HISTAMINE CONTENT INDUCED BY BRADYKININ*

Bradykinin (μg)	Bradykinin (nmoles)	cAMP (μmoles) 0	0·025	0·05	0·25
		Evans blue (μg/g)			
0·01	0·009	7·9			6·7 (16)
0·10	0·09	11·4	10·9 (5)	9·4 (18)	9·2 (20)
1·0	0·9	21·5			16·9 (21)
		Histamine (μg/g)			
1·0	0·9	77·0			1·5 (86)

* Rats were subcutaneously injected with each dose of bradykinin in combination with various doses of cAMP. Vascular permeability and cutaneous histamine content were assayed 30 min after the injections. Each value represents the mean of three experiments. The per cent inhibition is shown in parentheses.

Comparison of cAMP with various anti-inflammatory agents. As shown in Table 5, cAMP and epinephrine were as potent as hydrocortisone, three times as potent as

phenylbutazone, ten times as potent as indomethacin and about 100 times as potent as sodium salicylate and diphenhydramine in suppressing the PP_i-induced increase in vascular permeability. Among the compounds tested, cAMP and epinephrine were also the most effective inhibitors of the PP_i-induced increase in cutaneous histamine. As indicated also in Table 2, these compounds were more inhibitory to the PP_i-induced effects on vascular permeability than on cutaneous histamine.

TABLE 5. DOSES OF VARIOUS ANTI-INFLAMMATORY AGENTS FOR 50 PER CENT INHIBITION OF THE EFFECT OF 0·5 μmole PP_i*

Compounds	Doses required for 50 per cent inhibition	
	Vascular permeability increase (nmoles)	Histamine increase (μmoles)
Epinephrine	0·33	0·23
Cyclic 3′, 5′-AMP	0·40	0·25
Hydrocortisone	0·50	0·35
Phenylbutazone	1·0	0·45
Caffeine	2·0	0·50
Indomethacin	5·0	1·3
Sodium salicylate	38·0	2·5
Diphenhydramine	40·0	2·3

* Rats were subcutaneously injected either with 0·05 ml of 10 mM PP_i (controls) or with 0·05 ml of graded doses of each compound mixed with 0·5 μmole PP_i. Vascular permeability and cutaneous histamine content were measured 30 min after injection. Doses required for 50 per cent inhibition were calculated from the dose–response curves of each compound using two to three rats for each dose.

Effect of other nucleotides. As shown in Fig. 4, increases in vascular permeability and cutaneous histamine induced by PP_i were not effectively suppressed by adenine nucleotides other than cAMP, or by adenosine or adenine when administered at equimolar amounts with PP_i (0·5 μmole). cAMP suppressed ATP-induced inflammation as effectively as the PP_i-induced one.

5′-AMP suppressed the increase in cutaneous histamine induced by both PP_i and ATP, but had no significant effect on vascular permeability.

Among the cyclic 3′,5′-mononucleotides tested, only cAMP strongly inhibited the increases in vascular permeability and cutaneous histamine induced by PP_i (Table 6).

Effect of PP_i on histamine release from mast cells. Since it is assumed that histamine is mainly stored in mast cells, with some storage in leukocytes,[10,23] we investigated the effect of PP_i on histamine liberation from these cells. As shown in Table 7, a substantial amount of cellular histamine was released from mast cells incubated with 10 mM PP_i, pH 7·0, for 30 min at 37°, while only a little was released from leukocytes under similar conditions. We also observed that the total contents of histamine in both cells slightly increased during incubation with PP_i.

In addition to PP_i, ATP, a potent histamine releaser *in vivo* (Fig. 1), was also a strong releaser of histamine from isolated mast cells as reported by Diamant and Krüger.[7] Based on the dose required for a 50 per cent release of histamine from mast

TABLE 6. EFFECTS OF VARIOUS CYCLIC 3′, 5′-MONONUCLEOTIDES ON THE INCREASES IN VASCULAR PERMEABILITY AND CUTANEOUS HISTAMINE CONTENTS INDUCED BY PP_i*

Treatments	Vascular permeability (μg Evans blue/g)	Tissue histamine (μg/g)
PP_i	29·5	14·0
+ cAMP	8·5	5·0
+ cGMP	30·5	14·4
+ cUMP	22·5	13·2
+ cIMP	29·0	13·5
+ cCMP	26·0	13·5

* Rats were subcutaneously injected either with 0·05 ml of 10 mM PP_i, pH 7·2 (controls), or with 0·025 ml of 20 mM of each compound together with 0·025 ml of 20 mM PP_i, pH 7·2, and killed 30 min later for the assays. Each value represents the mean of three experiments.

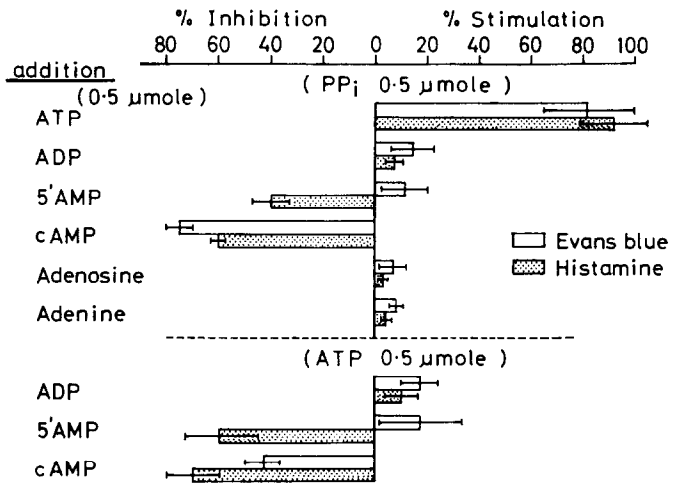

FIG. 4. Effects of adenine nucleotides on the changes in vascular permeability and cutaneous histamine induced by PP_i and ATP. Groups of six rats were injected with 0·05 ml of 10 mM PP_i or ATP (controls), or with 0·025 ml each of the compounds (20 mM) to be tested together with 0·025 ml of 20 mM PP_i or ATP. After 30 min, the rats were decapitated and vascular permeability and cutaneous histamine were assayed as described in the text.

cells, ATP was about 35 times as potent as PP_i (Fig. 5 inset, PP_i 0·7 μmole/ml = ATP 0·02 μmole/ml; the corresponding dose of compound 48/80 for 50 per cent release under the same conditions was 0·4 μg/ml). On the other hand, P_i, 5′-AMP, cAMP, and complexing agents like EDTA and citrate were not effective in this respect. (Fig. 5).

In contrast to the results reported by Diamant and Krüger,[7] the Ca^{2+} requirement for ATP-induced histamine release from mast cells was not very strict. Also, no significant difference between the effects of Ca^{2+} and Mg^{2+} was observed under our experimental conditions. In the absence of these metallic ions, histamine release induced

TABLE 7. EFFECT OF PP_i ON HISTAMINE RELEASE FROM ISOLATED LEUKOCYTES AND MAST CELLS OF RATS*

Cells	Origin	No. of samples	Histamine: Total (total, intact)† ($\mu g/10^5$ cells ± S.E.)		Histamine: Released by PP_i ($\mu g/10^5$cells ± S.E.)	PP_i (%)
Leukocytes	Blood	6	0·43 ± 0·08	(0·30 ± 0·03)†	0·03 ± 0·01	(7)
	Peritoneal exudate	6	0·50 ± 0·04	(0·41 ± 0·02)†	0·05 ± 0·01	(10)
Mast cells	Peritoneal fluid	6	3·96 ± 0·22	(2·36 ± 0·28)†	2·68 ± 0·28	(68)

* Leukocytes or mast cells (ca. 10^5 cells) were suspended in 1 ml of the incubation medium containing 10 mM PP_i for 30 min at 37°. The reaction was stopped by cooling the mixture to ice-cold. After light centrifugation, histamine in the supernatant fraction was assayed by the method described in Methods and Materials.

† Values in parentheses are total contents of histamine in non-incubated intact leukocytes or mast cells. These increases in total histamine after the incubation with PP_i were statistically significant ($P < 0·05$).

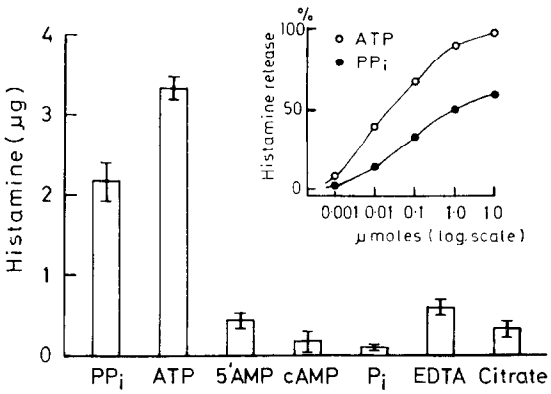

FIG. 5. Effect of PP_i and various other compounds on histamine release from isolated mast cells of rats. Mast cells (5×10^4 cells) were suspended in 1 ml of the incubation medium, containing 1 μmole each of the compounds to be tested, and incubated tor 30 min at 37°. The reaction was stopped by cooling the mixture to ice-cold. After centrifugation, histamine in the supernatant fraction was assayed as described under Methods and Materials.

by both PP_i and ATP (1 μmole each) decreased to about 65 per cent of their optimal values (PP_i, ca. 65 per cent release of the total histamine; ATP, ca. 80 per cent release) obtained in the presence of 0·9 mM Ca^{2+} or Mg^{2+}. At higher concentrations up to 5 mM, these metallic ions did not significantly alter the release of histamine induced by both PP_i and ATP.

Effect of cAMP and 5'-AMP on histamine release from mast cells induced by PP_i, ATP and compound 48/80. cAMP, a potent inhibitor of PP_i-induced inflammation *in vivo*, was also effective in suppressing histamine release from mast cells induced by PP_i, as well as by ATP and compound 48/80. In addition, 5'-AMP, which inhibited the increase in cutaneous histamine induced by PP_i and ATP *in vivo*, was as effective as cAMP in inhibiting histamine release *in vitro* by PP_i and ATP. However, 5'-AMP was not very inhibitory to the effects of compound 48/80 (Table 8).

TABLE 8. EFFECTS OF cAMP AND 5′-AMP ON HISTAMINE RELEASE FROM MAST CELLS INDUCED BY PP_i, ATP and COMPOUND 48/80*

Treatment	Controls	Histamine released from 10^5 cells (% release ± S.E.) cAMP (μmoles/ml) 0·01	0·1	1·0	5′-AMP (μmoles/ml) 0·01	0·1	1·0
PP_i(1 μmole)	62·5 ± 2·1	38·7 ± 1·4	30·0 ± 1·6	19·2 ± 1·1	42·5 ± 0·8	33·3 ± 0·3	20·0 ± 1·5
ATP (0·1 μmole)	89·3 ± 3·3	63·3 ± 1·9	58·3 ± 2·1	41·7 ± 1·5	66·7 ± 2·3	52·5 ± 1·2	35·0 ± 1·1
Compound 48/80 (1 μg)	77·5 ± 1·7	45·8 ± 1·9	37·4 ± 2·4	25·0 ± 0·3	66·7 ± 1·7	59·2 ± 2·5	55·8 ± 0·5

* Isolated mast cells (10^5 cells) were suspended in 1 ml of the medium and incubated with PP_i (1 μmole), ATP (0·1 μmole) or compound 48/80 (1 μg) in the presence of various amounts of cAMP or 5′-AMP for 30 min at 37°. Total histamine was 3·74 μg/10^5 cells.

Histamine release from mast cells induced by ATP and compound 48/80 proceeded rapidly,[24,25] reaching optimal values in about 5–10 min, while PP_i-induced histamine release slowly approached its optimal value in about 30 min. Therefore, in the present studies, all comparisons were made with 30-min incubations.

As observed in experiments *in vivo*, there was no essential difference between the inhibitory effects of cAMP and $N^6,O^{2'}$-dibutyryl cAMP on histamine release from isolated mast cells.

Furthermore, other nucleotides which were not effective inhibitors for the inflammatory effects of PP_i or ATP were also not significantly effective in suppressing the histamine release from mast cells.

DISCUSSION

PP_i and cAMP. Our observations indicate that PP_i is an inflammatory agent with several interesting characteristics.

First, the change in vascular permeability induced by PP_i in rats was immediate and monophasic, but not biphasic as observed in most experimental inflammations induced by various phlogistins,[15–20] and no delayed response was observed even 8 hr after PP_i injection. Although, according to Wilhelm,[26] an immediate and transient response is the rule in guinea pigs and rabbits in various types of experimental injuries, it may be feeble or absent in rats.

Second, PP_i-induced inflammation is probably mediated by endogenous histamine, because the time course of the change in vascular permeability correlated well with that of the increase in cutaneous histamine. Also, both responses to PP_i were suppressed by the simultaneous injection of diphenhydramine or by pretreatment of animals with compound 48/80.

We also examined the possibility of elevated secretion of catecholamines, since rats displayed apparent acute pain reactions at the time of PP_i injection. However, these shock reactions did not seem to be directly related to catecholamine secretion, because catecholamine contents in the blood and adrenals were not altered during the first 60 min after PP_i injection. In addition, prolonged administration of PP_i caused no significant changes in blood glucose.

Third, cAMP was an effective inhibitor of both increases in vascular permeability and cutaneous histamine induced by PP_i. Except for 5'-AMP, which fairly well inhibited the increase in cutaneous histamine induced by PP_i, no other adenine or cyclic nucleotides had such potent suppressive effects.

Furthermore, cAMP was as effective as epinephrine and hydrocortisone in suppressing both of the effects induced by PP_i, and was much more effective than non-steroidal anti-inflammatory drugs. Probably epinephrine suppresses the PP_i-induced vascular permeability change by its well known vasoconstricting activity, and it inhibits histamine increase by activating adenyl cyclase, which elevates the tissue concentration of cAMP.[27] Methylxanthines seem to suppress PP_i effects by inhibiting cAMP phosphodiesterase.[5] In contrast to epinephrine, cAMP does not seem to have a marked effect on the circulatory system. Kishimoto and Shimizu[28] reported that the intravenous injection of 1–5 mg cAMP/kg in anesthetized dogs showed no influence on systemic blood pressure and that, at a dosage of 10 mg/kg, this nucleotide caused a persistent slight depression of blood pressure.

Therefore, vasoconstriction does not appear to be related to the inhibitory effect of cAMP on PP_i-induced changes in vascular permeability.

Interestingly, hydrocortisone, which has little anti-anaphylactic activity,[29] suppressed PP_i-induced inflammation as effectively as it did capillary permeability increase and leukocytic diapedesis in thermal injury.[30,31] This may indicate that, in spite of acute pain reactions observed at the time of its administration, PP_i-induced inflammation is not of an anaphylactic type involving a certain antigen–antibody reaction.

Fourth, it seems unlikely that a kinin-like substance participates in the process of PP_i-induced inflammation, because cAMP effectively inhibited the increase in vascular permeability induced by PP_i or exogenous histamine, but not that by bradykinin, in spite of the marked suppressive effect of cAMP on the increase in cutaneous histamine induced by bradykinin.

Recently Baumgarten *et al.*[32] suggested the presence of two receptor sites in vascular endothelium cells, one specific toward bradykinin, the other specific toward both bradykinin and histamine. If so, cAMP might specifically inhibit the latter, but not the former site.

However, the effect of kinin released *in vivo* may differ from that of kinin injected. Furthermore, the acute pain reaction observed at the time of PP_i injection may be produced by much smaller quantities of kinin than that required for the vascular permeability changes, depending upon the proximity to nerve endings. This possibility must be considered also in light of the work of Lim[33] on pain.

Similarly, the role of serotonin cannot be ruled out completely, because the vascular permeability change induced by exogenous serotonin was suppressed by epinephrine, cAMP and methylxanthines, the suppressors of histamine-induced effects (Table 3).

From these reasons, it is still possible that multiple mediators are involved in the PP_i-induced inflammation.

Fifth, we also established that PP_i released histamine from isolated mast cells as did compound 48/80[25] and ATP,[7,25] but did not significantly release it from isolated leukocytes. Total histamine contents in mast cells and leukocytes slightly increased during incubation with PP_i, indicating a possible elevation of histamine formation by this agent (Table 7). However, *in vivo*, mobilization of histamine from other sites

by PP_i or ATP after remote injury, as reported by Geiringer and Hardwick,[34] is also another possibility. More studies on this, as well as on the enzyme systems for histamine synthesis and degradation, and its releasing mechanism are necessary for the elucidation of the exact mechanism by which tissue histamine is elevated.

As in PP_i-induced inflammation *in vivo*, cAMP, as well as 5′-AMP, effectively suppressed histamine release from mast cells, induced by PP_i *in vitro*.

PP_i and ATP. In these studies, we observed that the inflammatory effects of PP_i resembled those of ATP in several respects: (1) in the monophasic time courees of the changes in both vascular permeability and cutaneous histamine; (2) in the inhibition of the changes in vascular permeability induced by these compounds by pretreating rats with compound 48/80 or by simultaneous administration of an antihistamine; (3) in the effective inhibition of the changes in both vascular permeability and cutaneous histamine by cAMP; (4) in the inhibition of the increase in cutaneous histamine by 5′-AMP; and (5) in the induction of histamine release from mast cells and its inhibition by both cAMP and 5′-AMP.

On the other hand, the main difference between the two compounds is that ATP was more potent than PP_i in increasing cutaneous histamine *in vivo* and in releasing histamine from isolated mast cells. The latter effect of ATP was slightly more sensitive to Ca^{2+} than that of PP_i and optimal release by ATP in the presence of Ca^{2+} was higher than that by PP_i. In addition, rats displayed apparent acute pain reactions only at the time of PP_i administration. Effects of PP_i on the nervous system awaits further investigation.

cAMP and 5′-AMP. cAMP suppressed the increases in both vascular permeability and cutaneous histamine induced by PP_i or ATP, while 5′-AMP inhibited only the increase in cutaneous histamine. In contrast, 5′-AMP was as effective as cAMP in inhibiting the histamine release from isolated mast cells induced by both PP_i and ATP. This suggests that ATP and 5′-AMP are involved in an ATP–ATPase system for the release of a secretory product from various granules, as suggested by Poisner and Douglas,[35] or in a similar system for the release of histamine from mast cells, as suggested by Diamant.[24] 5′-AMP may suppress histamine release by inhibiting the ATPase of the storage cells.

cAMP may be hydrolyzed to 5′-AMP before it inhibits the ATP–ATPase system for the liberation of histamine. However, it is difficult to explain how cAMP can inhibit the PP_i-induced changes in both vascular permeability and cutaneous histamine, while 5′-AMP can only inhibit the increase in cutaneous histamine. Some of our results suggest that cAMP acts *per se* to suppress the PP_i-induced effects: (1) There is practically no difference between the suppressive effects of cAMP and N^6,$O^{2'}$-dibutyryl cAMP, in spite of the fact that the latter compound is known to have a greater resistance to cAMP phosphodiesterase and a higher penetration rate through the cell membrane. (2) Suppressive effects of cAMP on the PP_i-induced changes in both vascular permeability and cutaneous histamine were slightly but significantly augmented by addition of methylxanthines, inhibitors of cAMP phosphodiesterase.

Ryan and Heidrick[36] observed that dibutyryl cAMP was less effective than cAMP in inhibiting the growth of cultured L-cells, and they suspected the absence of the enzyme to convert the dibutyryl derivative to cAMP in the L-cells. A similar conversion of dibutyryl cAMP might be necessary also in our case.

If the adenyl cyclase reaction in animal tissues is readily reversible, as is the enzyme

reaction in *Brevibacterium liquefaciens* (ATP $\rightleftarrows$ cAMP + PP_i),[37] then exogenous PP_i, if not hydrolyzed to P_i, might react with endogenous cAMP to form ATP, which in turn is available to the ATP–ATPase system for histamine release. However, inhibition of PP_i-induced inflammation by simultaneous administration of PP_i and cAMP, or inhibition of PP_i-induced histamine release by cAMP is not explainable by this reversible reaction.

Since, in our research, the effects of PP_i or ATP were antagonized by cAMP *in vivo* and *in vitro*, the situation is different from that of cAMP-dependent protein kinase, which activates a particular enzyme with ATP,[38] where both cAMP and ATP function cooperatively.

Recently, Rasmussen[39] postulated a control mechanism with cAMP and Ca^{2+} for various types of cellular activities. However, histamine release from isolated mast cells was not strictly Ca^{2+} dependent in our experimental conditions.

Since there were similarities between the inflammatory effects of PP_i and ATP, we need to know the nature of ATP-induced inflammation to understand the effects of PP_i and cAMP in the PP_i-induced inflammatory process, which is probably mediated by multiple mediators.

REFERENCES

1. K. TOMITA, J. W. YOUNG and H. A. LARDY, *Gunma Symp. Endocr.* **5,** 85 (1968).
2. W. G. SPECTOR and D. A. WILLOUGHBY, *J. Path. Bact.* **80,** 271 (1960).
3. R. O. BROWN and G. B. WEST, *J. Pharm. Pharmac.* **17,** 119 (1965).
4. E. W. SUTHERLAND and G. A. ROBISON, *Pharmac. Rev.* **18,** 145 (1966).
5. R. W. BUTCHER and E. W. SUTHERLAND, *J. biol. Chem.* **237,** 1244 (1962).
6. L. M. LICHTENSTEIN and S. MARGOLIS, *Science, N.Y.* **161,** 902 (1968).
7. B. DIAMANT and P. G. KRÜGER, *Acta physiol. scand.* **71,** 291 (1967).
8. V. L. BEACH and B. G. STEINETZ, *J. Pharmac. exp. Ther.* **131,** 400 (1961).
9. P. A. SHORE, A. BURKHALTER and V. H. COHN, JR., *J. Pharmac. exp. Ther.* **127,** 182 (1959).
10. L. M. LICHTENSTEIN and A. G. OSLER, *J. exp. Med.* **120,** 507 (1964).
11. H. STÄHELIN, E. SUTER and M. L. KARNOVSKY, *J. exp. Med.* **104,** 121 (1956).
12. N. CHAKRAVARTY and E. ZEUTHEN, *J. Cell Biol.* **25,** 113 (1965).
13. U. S. VON EULER and I. FLODING, *Acta physiol. scand.* **33,** suppl. 118, 45 (1955).
14. M. SOMOGYI, *J. biol. Chem.* **160,** 61 (1945).
15. H. HAYASHI, M. YOSHINAGA, M. KOONO, H. MIYOSHI and M. MATSUMURA, *Br. J. exp. Path.* **45,** 419 (1964).
16. D. L. WILHELM and B. MASON, *Br. J. exp. Path.* **47,** 487 (1960).
17. W. G. SPECTOR and D. A. WILLOUGHBY, *Nature, Lond.* **182,** 949 (1958).
18. J. F. BURKE and A. A. MILES, *J. Path. Bact.* **76,** 1 (1958).
19. R. NITTA, *Kumamoto med. J.* **18,** 72 (1965).
20. W. G. SPECTOR and D. A. WILLOUGHBY, *J. Path. Bact.* **77,** 1 (1959).
21. W. FELDBERG and J. TALESNIK, *J. Physiol., Lond.* **120,** 550 (1953).
22. J. R. PARRATT and G. B. WEST, *J. Physiol., Lond.* **137,** 179 (1957).
23. L. M. LICHTENSTEIN, P. S. NORMAN, W. L. WINKENWERDER and A. G. OSLER, *J. clin. Invest.* **45,** 1126 (1966).
24. B. DIAMANT, *Int. Archs Allergy appl. Immun.* **36,** 3 (1969).
25. K. SAEKI, *Jap. J. Pharmac.* **14,** 375 (1964).
26. D. L. WILHELM, *Pharmac. Rev.* **14,** 251 (1962).
27. E. W. SUTHERLAND, G. A. ROBISON and R. W. BUTCHER, *Circulation* **37,** 279 (1968).
28. T. KISHIMOTO and H. SHIMIZU, *J. pharm. Soc. Japan* **63,** 461 (1967).
29. P. GOADBY and W. G. SMITH, *J. Pharm. Pharmac.* **16,** 108 (1964).
30. V. MENKIN, *Br. J. exp. Path.* **34,** 412 (1953).
31. F. ALLISON, JR., M. R. SMITH and W. B. WOOD, JR., *J. exp. Med.* **102,** 669 (1955).
32. A. BAUMGARTEN, G. J. H. MELROSE and W. J. VAGG, *J. Physiol., Lond.* **208,** 669 (1970).
33. R. K. S. LIM, *A. Rev. Physiol.* **32,** 269 (1970).
34. E. GEIRINGER and D. C. HARDWICK, *J. Physiol., Lond.* **119,** 410 (1953).
35. A. M. POISNER and W. W. DOUGLAS, *Molec. Pharmac.* **4,** 531 (1968).

36. W. L. RYAN and M. L. HEIDRICK, *Science, N.Y.* **162,** 1484 (1968).
37. P. GREENGARD, O. HAYASHI and S. P. COLOWICK, *Fedn Proc.* **28,** 467 (1969).
38. T. R. SODERLING, J. P. HICKENBOTTOM, E. M. REIMANN, F. L. HUNKELER, D. A. WALSH and E. G. KREBS, *J. biol. Chem.* **245,** 6317 (1970).
39. H. RASMUSSEN, *Science, N.Y.* **170,** 6317 (1970).