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Pharmacological modulation of platelet-activating factor (PAF) release from rabbit leucocytes

I. ROLE OF cAMP

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Summary. Basophil-rich rabbit leucocytes sensitized by anti-horseradish peroxidase antibodies released platelet-activating factor (PAF) and histamine upon exposure to the specific antigen. This release was preceded and accompanied by a sharp decrease in the intracellular concentration of cyclic AMP. Isoproterenol, a β-adrenergic agent, and theophylline, a phosphodiesterase inhibitor, used individually or in combination, increased the intracellular concentration of cyclic AMP and inhibited the release of both PAF and histamine. Propranolol, a β-adrenergic blocking agent, suppressed the effect of isoproterenol on cyclic AMP level and mediator release. Dibutyryl cyclic AMP, an alkylated derivative of cyclic AMP, inhibited PAF and histamine release. These results indicate that cyclic AMP, which is known to control the release of other mediators of immediate hypersensitivity, also regulates the release of PAF. Histamine and PAF followed one another closely in all of our release or inhibition experiments, bringing more evidence for the basophil origin of PAF.

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Abbreviations: PAF, platelet-activating factor; SRS-A, slow-reacting substance of anaphylaxis; cAMP, cyclinc adenosine 3′-5′ monophosphate; PGE_1, prostaglandin E_1; ConA, concanavalin A; HRP, horseradish peroxidase; BSA, bovine serum albumin; TG, Tyrode's buffer with 0·25% gelatin; TG no Ca^{2+} no Mg^{2+}, TG without calcium and magnesium; Tris, tris-(hydroxymethyl)-aminomethane; TT-BSA, Tris-buffered Tyrode's with 0·25% BSA; TT-BSA no Ca^{2+} no Mg^{2+}, TT-BSA without calcium and magnesium; TBS, Tris-buffered saline; BCC, buffy-coat cells; TCA, trichloracetic acid; ^3H-cAMP, tritiated cyclic adenosine 3′-5′-monophosphate.

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INTRODUCTION

The release of anaphylactic mediators such as histamine and slow-reacting substance of anaphylaxis (SRS-A) from basophils and from mastocytes, is regulated *in vitro* by cyclic adenosine 3′-5′ monophosphate (cAMP) (Lichtenstein & Margolis, 1968; Lichtenstein & De Benardo, 1971; Bourne, Lichtenstein & Melmon, 1972; Orange, Austen & Austen, 1971; Ishizaka, Ishizaka, Orange & Austen, 1971; Kaliner & Austen, 1974). β-Adrenergic agonists, phosphodiesterase inhibitors, and adenyl cyclase activators, increase the intracellular levels of cAMP, and inhibit the release of mediators of anaphylaxis (Lichtenstein & Margolis, 1968; Lichtenstein & De Benardo, 1971; Bourne *et al.*, 1972; Orange *et al.*, 1971; Ishizaka *et al.*, 1971; Kaliner & Austen, 1974; Bourne, Melmon & Lichtenstein, 1971). Dibutyryl cAMP which mimics the effects of the cAMP is also inhibitory. These agents decrease the antigen-induced release of histamine and SRS-A from human leucocytes (Lichtenstein & Mar-

golis, 1968; Lichtenstein & De Benardo, 1971; Bourne *et al.*, 1972; Bourne *et al.*, 1971), human (Orange *et al.*, 1971) and monkey (Ishizaka *et al.*, 1971) lung, and rat mastocytes (Kaliner & Austen, 1974). PGE_1 and methylxanthines have been shown to decrease the release of histamine from rat mastocytes treated with compound 48/80 (Sullivan, Parker, Stenson & Parker, 1975a, b). Cyclic-AMP has been demonstrated to control the mediator release from human leucocytes stimulated by concanavalin A (ConA) (Siraganian & Siraganian, 1974) and C_{5a} (Hook & Siraganian, 1977). Intracellular levels of cAMP decrease during histamine release in rat mast cells stimulated by either rabbit anti-Fab portion of rat IgGa (Kaliner & Austen, 1974), or by compound 48/80 (Gillespie, 1973; Sullivan *et al.*, 1975a, b), a fall which depends on the activation of a phosphodiesterase (Sullivan & Parker, 1973). A transient fall in basal adenyl cyclase activity was also demonstrated in guinea-pig lung during antigen-induced histamine release (Hitchcock, 1977).

These results link the cAMP system with the mechanism of mediator release in immediate hypersensitivity, but they do not explain the variation of intracellular cAMP during the antigenic stimulation of target cells. Furthermore, the role of cAMP in the regulation of the newly discovered mediator, platelet-activating factor (PAF) (Siraganian & Osler, 1971; Benveniste, Henson & Cochrane, 1972; Benveniste, 1974), has not yet been described. PAF is a phospholipid (Benveniste, Le Couedic, Polonsky & Tencé, 1977b) mediator of anaphylaxis released from human (Camussi, Mencia-Huerta & Benveniste, 1977), rabbit (Benveniste *et al.*, 1972), and hog (Benveniste, 1974) peripheral leucocytes, most probably basophils as shown by the dependency on IgE antibodies on the release from rabbit leucocytes (Benveniste, *et al.*, 1972) and from rat and mouse peritoneal macrophages (Benveniste & Mencia-Huerta, 1978; Mencia-Huerta, & Benveniste, 1979). It is a potent platelet-aggregating and releasing agent. It has been implicated in the initiation of immune complex deposition in acute serum sickness in rabbits (Benveniste, Egido & Gutierrez-Millet, 1976). Recently, it has been identified as a 1-0-alkyl-2-acetyl-3-glyceryl-phosphorylcholine (Benveniste *et al.*, 1979; Demopoulos, Pinckard & Hanahan, 1979), and therefore termed PAF-acether.

The aim of this work was to explore the antigen dependent variations of cAMP levels in rabbit leucocytes and their relation with PAF and histamine release.

MATERIALS AND METHODS

Animals

New Zealand White male and female rabbits, weighing 1·8–2·2 kg, were immunized by two subcutaneous injections of 20 mg and 10 mg horseradish peroxidase (HRP) in physiological saline at 3 week intervals. In rabbits, this technique induces homocytotropic anti-HRP antibodies with passive cutaneous anaphylaxis titres of 1/40 to 1/80, sufficient to highly sensitize circulating basophils (Benveniste *et al.*, 1972; Benveniste *et al.*, 1977). Untreated rabbits were used as donors for platelets.

Chemicals

The following reagents were used: isoproterenol HCl, DL-propranolol HCl, theophylline, HRP type II, adenosine 3′-5′ cyclic monophosphoric acid sodium salt, $^6N^2O$ dibutyryl adenosine 3′-5′ cyclic monophosphoric acid grade II, arachidonic acid, indomethacin (Sigma Chemical Company, St Louis, MO), gelatin (Difco Lab. Detroit, MI), bovine serum albumin fraction V (BSA) (Pharmindustrie, Clichy, France), phospholipase A_2 from hog pancreas (Boehringer Mannheim, G.F.R.), [^{3}H]-adenosine 3′-5′ cyclic monophosphoric acid ammonium salt (Commissariat à l'Energie Atomique, Gif-sur-Yvette, France), 2,5-diphenyloxazole scintillation grade 1,4-bis-[2(4-methyl-5-phenyloxazolyl)]-benzene scintillation grade (Packard Instrument Inc. Company, Warrenville, IL). Chemicals for buffers were reagent grade from Merck (Darmstadt, G.F.R.), except for tris-(hydroxymethyl)-aminomethane (Tris) which was purchased from Sigma.

Buffers

The following buffers were prepared: Tyrode's gelatin (TG); KCl $2{\cdot}6 \times 10^{-3}$ M; $MgCl_26H_2O$ 1×10^{-3} M; NaCl $1{\cdot}37 \times 10^{-1}$ M; $NaHCO_3$ 1×10^{-2} M; $CaCl_26H_2O$ $1{\cdot}3 \times 10^{-3}$ M, 2·5 g/l gelatin: TG no Ca^{2+} no Mg^{2+}, TG from which Ca^{2+} and Mg^{2+} have been omitted; Tyrode's buffered with 1×10^{-2} M Tris instead of bicarbonate and 2·5 g/l BSA instead of gelatin (TT-BSA); TT-BSA no Ca^{2+} no Mg^{2+}, same medium from which Ca^{2+} and Mg^{2+} have been omitted. Dialysis of samples before dosage of PAF was performed against Tris 1×10^{-2} M and NaCl $1{\cdot}4 \times 10^{-1}$ M buffer pH 7·2 (TBS). For cAMP assay, we used a sodium acetate buffer 5×10^{-2} M pH 4 and a sodium phosphate buffer 1×10^{-2} M pH 6.

Preparation of cells

Washed rabbit platelets and buffy-coat cells (BCC)

were prepared exactly as previously described (Benveniste *et al.*, 1972). BCC contained 80–90% lymphomononuclear cells, 0–4% polymorphonuclear neutrophils, and 5–10% basophils.

Release and determination of PAF and histamine from BCC

BCC (1×10^7) from immunized rabbits were incubated at 37° for 5 min in 1 ml TT-BSA with or without the pharmacological agents and with the concentration of antigen that, in individual rabbits, gave the highest percentage of basophil degranulation (Benveniste *et al.*, 1977b). The reaction was stopped by centrifugation at 800 ***g*** at 0° for 10 min, except in the kinetic studies, where ethylene-diamine-tetra-acetate (EDTA) (5×10^{-3} M for PAF and histamine assays), and freezing by liquid nitrogen for cAMP, were used. After centrifugation, the cell-free supernatants were dialysed against TBS to remove adenosine diphosphate and the pharmacological agents which could interfere with the the platelet aggregation. Concentration of Tris in TBS were shown not to be inhibitory to PAF-induced platelet aggregation. The total amount of releasable PAF was obtained by incubating BCC overnight in TT-BSA at pH 10·6 (Benveniste, 1974).

PAF was detected by aggregation of 5×10^7 washed rabbit platelets (aggregometer: Icare, Marseille, France) stirred at 900 r.p.m. in 300 μl TG (Benveniste, 1974). Maximum aggregation was obtained by adding 0·1 unit/ml of thrombin to the platelets. PAF activity was expressed in arbitrary units/ml, using the following formula:

$$\frac{1}{\mu\text{l PAF for 50\% of maximal aggregation}} \times 1000.$$

This technique gave reproducible results above 8 units/ml of PAF. The influence of pharmacological agents on the PAF-induced aggregation was tested by adding them at various concentrations in the aggregometer tubes 1 min or immediately before the addition of PAF.

PAF was identified by three criteria (Benveniste *et al.*, 1977a, b): (1) persistence of platelet aggregation in the presence of 5×10^{-6} M indomethacin which inhibits the aggregation produced by 1×10^{-5} M arachidonic acid; (2) hydrolysis of PAF by phospholipase A_2 which completely suppresses its aggregating effect; (3) thin layer silica-gel chromatography (TLC) with chloroform–methanol–water (70:35:7) as solvent. PAF exhibited an Rf index of 0·35. These identification procedures were carried out as follows: all samples that exhibited a platelet-aggregating activity were, once measured, pooled together and studied by TLC PAF was eluted from the gel and a sample of the elution fluid was incubated 10 min at 37° with 10 μg/ml phospholipase A_2 with or without EDTA exactly as described (Benveniste *et al.*, 1977a, b).

Histamine was assessed by a spectrofluorometric procedure (Ruff, Saindelle, Dutripan & Parrot, 1967) with a minimum sensitivity of 20 ng/ml. Total histamine was extracted by boiling the cell suspension for 5 min.

Cyclic AMP extraction and assay

The cells were separated from the medium by centrifugation at 800 ***g***, at 0°, for 10 min. The pellet was resuspended in 500 μl of 5% trichloroacetic acid (TCA) followed by 50 μl of 1 N HCl. After centrifugation (800 ***g***, 10 min, room temperature) supernatants were transferred to glass tubes and TCA was extracted with ether. The samples were lyophilized and 500 μl of sodium acetate buffer was added. Forty microlitres of the samples that had been incubated with antigen and the cAMP-augmenting pharmacological agents, and 100 μl of the samples incubated only with antigen, were used for the assay. Cyclic AMP was determined according to the protein binding assay of Gilman (1970). The cAMP-binding protein and protein kinase inhibitor were prepared from bovine striated muscle as described by the same author. The amount of cAMP was calculated by comparing inhibition of [^{3}H]-cAMP binding with a standard curve of inhibition by known concentrations of cAMP (from 0·0625 pM to 5 pM) with an inhibitory activity between 15 and 80%. All assays were run in duplicate.

RESULTS

Release of PAF and histamine and changes of cellular cAMP with antigenic stimulation

When BCC were incubated with increasing doses of HRP, PAF and histamine were released in a dose–response fashion. Above 0·01 μg/ml HRP, the intracellular level of cAMP decreased in a dose–response fashion as well (Table 1). The maximal decrease of cAMP (60·4 ± 16·0% of the amount found in BCC not exposed to antigenic stimulation) was seen at the antigen concentration (100 μg/ml) which produced the maximal release of PAF and histamine. The amount of intracellular cAMP was maximal in unstimulated

Table 1. Dose-dependent release of PAF and histamine and cellular cAMP levels*

HRP μg/ml)	PAF†	Histamine†	cAMP
0	0	6·3±3·2	21·0±3·1‡
0·01	0	8·9±6·1	23·7±5·3 NS
0·1	0	11·7±3·8	19·7±3·9 NS
1	15·3±13·8	26·0±16·0	12·1±3·1 ***
10	39·7±6·6	44·0±9·6	9·8±2·5 ***
100	60·3±13·4	60·1±3·0	8·3±3·6 ***

* 1×10^7 sensitized rabbit BCC in 1 ml TT-BSA were incubated with HRP at 37° for 5 min.

† Results are expressed as mean ± 1 SD (four experiments) of the percentage of release over the total amounts of PAF: 810, 600, 1000, 970 units/ml; and histamine: 960, 4120, 2100, 1800 ng/ml.

‡ Mean ± 1 SD (four experiments) of intracellular concentration in pM. Statistical analysis of differences with the cAMP concentrations in BCC without antigen (*t* test): NS not significant; *** $P < 0.001$.

BCC where no detectable PAF and very little histamine were released.

The kinetics of cAMP levels and of PAF and histamine release were next investigated (Fig. 1). In these experiments, the concentration of antigen added to the test tubes varied from 1 to 100 μg/ml according to the degree of BCC sensitivity (see Methods). PAF and histamine release was preceded by an immediate decrease in cAMP which reached its lowest concentration (45·5±5·9% decrease from starting levels) 50 s after addition of antigen and kept at this level throughout the incubation period. At 25 s, histamine release was 7·6±5·3%, whereas no PAF was detectable; thereafter, the release kinetics of the two mediators were parallel, and did not show statistically significant differences throughout the time-course.

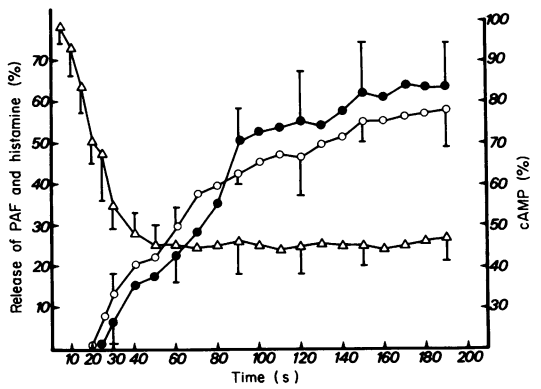

Figure 1. Kinetics of antigen-induced release of PAF (●), histamine (○), and changes in cAMP intracellular levels (△). Sensitized rabbit BCC (1×10^7) were incubated in 1 ml TT-BSA at 37° for 5 min with optimal concentration of HRP. Each point represents the mean ± 1 SD of five experiments. Only some representative SD are given in the figure. Percentages were calculated over the total amounts of PAF: 900, 510, 666, 630, 840 units/ml; and histamine: 2850, 1980, 2870, 3030, 1000 ng/ml; and over the cAMP concentration in 1×10^7 cells incubated without antigen: 19·0, 17·8, 28·5, 23·6 pM. Spontaneous histamine release at 200 s was 3·4±4·0%, and was not subtracted from the active release. The curves of histamine and PAF release were not statistically different (*t* test), except between 20 s and 25 s ($P < 0.001$).

The effect of pharmacological agents on cAMP levels and on the release of PAF and histamine

Isoproterenol. Isoproterenol inhibited the antigen-induced release of PAF and histamine in a dose–response fashion (Fig. 2 and Table 2). The highest increase

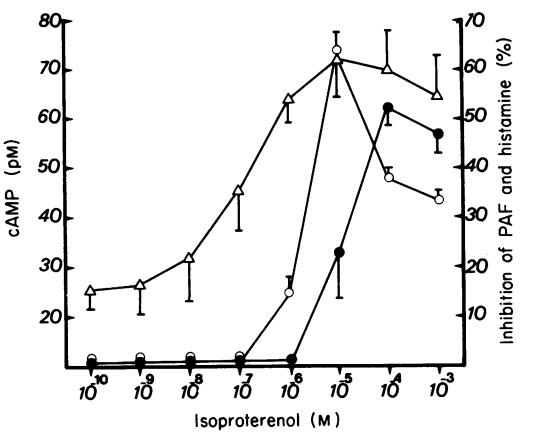

Figure 2. Effect of isoproterenol on antigen-induced release of PAF (●), histamine (○), and on cellular cAMP levels (△). Conditions for incubation of BCC were the same as in Fig. 1. Each point represents the mean ± 1SD of four experiments. Percentages of inhibition were calculated over values of PAF: 250, 230, 140, 500 units/ml; and histamine: 920, 1050, 480, 960 ng/ml, released by antigen alone. Spontaneous release of histamine and PAF was 6·4±2·1% and none, respectively. The intracellular cAMP in unstimulated BCC (1×10^7) was 26·0±3·4 pM. The release of PAF and histamine, in the presence of 1×10^{-6} to 1×10^{-3} M isoproterenol (histamine) and of 1×10^{-5} to 1×10^{-3} M isoproterenol (PAF), was statistically different (*t* test $P < 0.001$) from that with antigen alone or with the non-inhibitory concentrations of isoproterenol. There was no difference between the percentages of inhibition at 1×10^{-4} M versus 1×10^{-3} M isoproterenol. Differences in cAMP levels for control experiments (no isoproterenol and no antigen) were statistically significant ($P < 0.001$) to values recorded with 1×10^{-7} M to 1×10^{-3} M isoproterenol. There was no difference between 1×10^{-6} M, and 1×10^{-4} M isoproterenol.

Table 2. Effect of theophylline and propranolol on isoproterenol inhibition of antigen-induced release of PAF and histamine, and increase of cellular cAMP*

Drugs		Inhibition of PAF release (%)	Inhibition of histamine release (%)	Increase of cAMP (%)
Theophylline	1×10^{-4} M	5·2 ± 7·0†	2·4 ± 1·7	38·5 ± 10·6
Isoproterenol	1×10^{-3}	45·8 ± 6·1	33·5 ± 2·3	146·3 ± 7·3
Isoproterenol	1×10^{-4} M	54·1 ± 3·8	38·8 ± 2·5	170·0 ± 9·2
Isoproterenol	1×10^{-5} M	15·4 ± 4·7	62·4 ± 4·7	180·6 ± 12·6
Theophylline	1×10^{-4} M			
+ Isoproterenol	1×10^{-3} M	78·2 ± 8·2 $P < 0·01$	41·5 ± 5·7 $P < 0·05$	178·3 ± 5·7 $P < 0·01$
Theophylline	1×10^{-4} M			
+ Isoproterenol	1×10^{-4} M	91·6 ± 7·8 $P < 0·001$	54·3 ± 4·1 $P < 0·01$	237·3 ± 24·6 $P < 0·01$
Theophylline	1×10^{-4} M			
+ Isoproterenol	1×10^{-5} M	58·6 ± 10·4 $P < 0·01$	76·3 ± 4·5 $P < 0·05$	275·9 ± 2·2 $P < 0·001$
Propranolol	1×10^{-5} M	0	0·9 ± 1·1	0
Propranolol	1×10^{-5} M			
+ Isoproterenol	1×10^{-4} M	4·9 ± 3·5 $P < 0·001$	9·0 ± 5·1 $P < 0·001$	8·5 ± 4·6 $P < 0·001$
Propranolol	1×10^{-5} M			
+ Isoproterenol	1×10^{-5}	3·8 ± 2·1 $P < 0·001$	6·3 ± 3·2 $P < 0·001$	9·0 ± 10·6 $P < 0·001$

* 1×10^7 rabbit BCC were incubated in 1 ml TT-BSA at 37° for 5 min with 50 μg HRP.

† Percentages (mean ± 1 SD of four experiments) were calculated over values of PAF: 335, 730, 170, 600 units/ml; and histamine: 595, 1700, 1250, 1860 ng/ml, released by antigen alone, and over values of cAMP: 23·0, 21·1, 28·0, 29·0 pM in 10^7 cells incubated without antigen and pharmacological agents. Spontaneous release of histamine and PAF was 7·0 ± 4·7%, and none, respectively.

in cAMP was observed at 1×10^{-5} M isoproterenol: from 25·2 ± 2·3 to 72·1 ± 9·2 pM, an increase of 186% from the values at 1×10^{-10} M isoproterenol (Fig. 2). In this experiment, the level of cAMP in antigen-stimulated cells without isoproterenol was 17·6 ± 3·0 pM after 5 min, a decrease of 32% from unstimulated cells (26·0 ± 3·4 pM). The lowest concentrations of isoproterenol were inefficient in blocking histamine and PAF release but suppressed the drop in cAMP level noted under antigenic stimulation.

The most effective inhibition of the antigen-induced release of PAF (52·0 ± 3·6%) and histamine (63·4 ± 3·6%) was obtained with 1×10^{-4} M and 1×10^{-5} M isoproterenol, respectively.

Reversal of isoproterenol inhibition by propranolol. Isoproterenol at a concentration of 1×10^{-4} M inhibited histamine and PAF release by 38·8 ± 2·5% and 54·1 ± 3·8%, respectively, and increased the cAMP cellular levels of 170·0 ± 9·2%. These effects were completely suppressed by 1×10^{-5} M propranolol. By itself, propranolol neither enhanced the release of mediators nor modified cAMP levels (Table 2).

Theophylline. Theophylline, added to BCC in the presence of antigen, evoked an increase in cAMP and an inhibition of the antigen-induced release of PAF and histamine in a dose–response fashion (Fig. 3). Theophylline (1×10^{-6} M) reduced but did not suppress the antigen-induced drop in cAMP concentration from 24·2 ± 6·9 pM (level in unstimulated cells) to 16·4 ± 6·9 pM, a decrease of 33%. The highest concentration of theophylline increased cAMP to 63·4 ± 16·6 pM, an increase of 163% and inhibited the antigen-induced release of PAF and histamine 98·8 ± 1·6% and 78·1 ± 6·2%, respectively. At 1×10^{-3} M theophylline, the level of cAMP was 55·4 × 14·8 pM and the inhibition of PAF and histamine was 70·5 ± 2·7% and 44·6 ± 13·2%, respectively.

Synergy between theophylline and isoproterenol. A non-inhibitory dose of theophylline (1×10^{-4} M) accentuated the increase of cAMP and the isoproterenol-induced inhibition of the antigen-dependent release of PAF and histamine (Table 2). The enhancement of cAMP levels always coincided with decreased release of mediators, without correlation between the degree of cAMP enhancement and the percentage of release inhibition.

Dibutyryl cAMP. Addition of 1×10^{-2} M dibutyryl cAMP to BCC produced a dose–response inhibition of

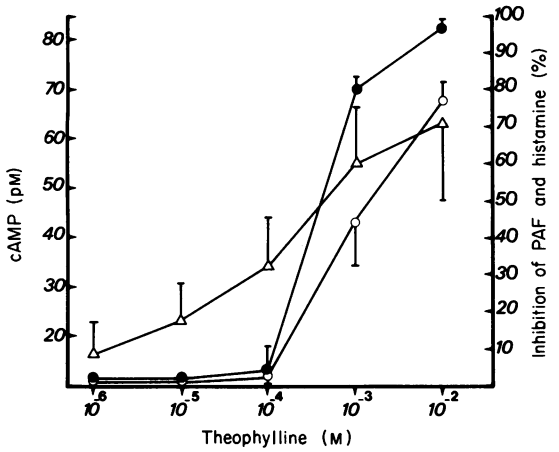

Figure 3. Effect of theophylline on antigen-induced release of PAF (•), histamine (○), and on cellular cAMP (△). Each point represents the mean ± 1 SD of five experiments. Percentages of inhibition were calculated over the values of PAF: 485, 500, 210, 500 units/ml; and histamine: 920, 1050, 1900, 610, 2050 ng/ml, released by antigen alone. Spontaneous release of histamine and PAF was 5·6 ± 2·2% and none, respectively. The intracellular cAMP in 1×10^7 unstimulated BCC was 24·2 ± 6·9 pM. The release of PAF and histamine in the presence of 1×10^{-3} and 1×10^{-2} M theophylline was statistically different (t test $P < 0.01$) from that with the antigen alone and with non-inhibiting concentrations of theophylline. The inhibition of histamine release differed between 1×10^{-2} M and 1×10^{-3} M theophylline, and from 1×10^{-3} M and 1×10^{-2} M theophylline ($P < 0.01$); there was no difference in cAMP levels between 1×10^{-3} M and 1×10^{-2} M theophylline.

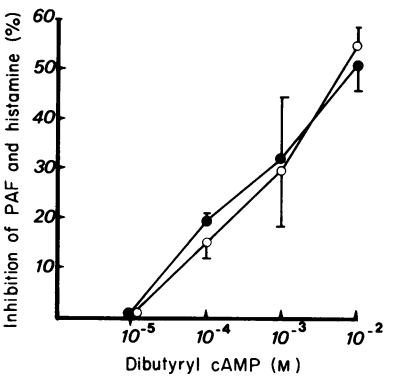

Figure 4. Effect of dibutyryl cAMP on antigen-induced release of PAF (•) and histamine (○). Each point represents the mean ± 1 SD of three experiments. Percentages of inhibition were calculated over the values of PAF: 238, 750, 510 units/ml; and histamine: 3240, 840, 1500 ng/ml, released by antigen alone. Spontaneous release of histamine and PAF was 7·1 + 2·3% and none, respectively. The release of PAF and histamine in the presence of 1×10^{-4} to 1×10^{-2} M dibutyryl cAMP was significantly different (t test $P < 0.001$) from that with antigen alone or antigen with 10^{-5} M dibutyryl cAMP.

the release of PAF (51·8 ± 5·5%) and histamine (55·3 ± 3·5%). There was no significant difference between the effect on PAF release as compared to histamine release (Fig. 4).

The effect of isoproterenol and theophylline on PAF-induced platelet aggregation

Isoproterenol and theophylline inhibited PAF-induced platelet aggregation. Isoproterenol (1×10^{-4} M) inhibited PAF aggregation by 70%. Concentrations of isoproterenol from 1×10^{-5} to 1×10^{-11} M and of theophylline from 1×10^{-4} to 1×10^{-8} M had no effect.

DISCUSSION

Our studies on semi-purified leucocytes from rabbits immunized so as to produce high titres of homocytotropic antibodies indicate that cAMP regulates the *in vitro* antigen-induced release of the two anaphylactic mediators, PAF and histamine. Existing evidence indicates that basophils are the source of PAF in rabbit blood (Siraganian & Osler, 1971; Benveniste *et al.*, 1972; Benveniste, 1974); the IgE-dependent mechanism is the main pathway of basophil activation and release of PAF and histamine in this species (Benveniste *et al.*, 1972). The parallelism of the release of PAF and histamine in our present studies is further evidence for the implication of basophils in the release of PAF. These cells are already a well known source of blood histamine (Pruzansky & Patterson, 1967; Galli, Dvorak & Dvorak, 1976). We have substantiated the modulating role of cAMP on histamine and PAF release by rabbit basophils with the following observations: (1) isoproterenol and theophylline (used alone or together) increased intracellular cAMP and inhibited the release of both mediators; (2) the release of PAF and histamine was preceded and accompanied by a decrease in cAMP. The maximal inhibition of PAF release by isoproterenol (52%) was reached at 1×10^{-4} M concentration, whereas 1×10^{-5} M was sufficient to inhibit maximally the release of histamine (63%). Such differences, as we noted for all other concentrations of isoproterenol, were probably indicative of different metabolic pathways and/or differences in the level of

the signal triggering the release of the mediators. However, in all cases, the highest inhibitory effect of isoproterenol and PAF release occurred when cAMP reached its maximum level (170%). Theophylline induced the highest increase in cAMP (163%) at 1×10^{-2} M, a concentration giving maximal inhibition of the antigen-induced release of PAF (98%) and histamine (78%).

The effect of PAF on platelet aggregation was inhibited by a dose of isoproterenol of 1×10^{-4} M but not by lower concentrations, results similar to those obtained by others (Henson & Oades, 1976). One to ten microlitres of supernatants in 300 μl of TG were used to measure PAF activity. At this dilution, the concentrations of isoproterenol (from 1×10^{-5} M to 1×10^{-11} M) or theophylline (from 1×10^{-4} M to 1×10^{-8} M), that were incubated with the platelets during the dosage of PAF, could not inhibit the PAF-induced aggregation. However, to avoid any interference, we removed the drugs before PAF assay by dialysing all BCC supernatants against TBS.

The inhibition of antigen-induced release of PAF and histamine by isoproterenol and theophylline is very comparable to that found by many authors on histamine and SRS-A release from various tissues or cell preparations (Lichtenstein & Margolis, 1968; Lichtenstein & De Benardo, 1971; Bourne *et al.*, 1972; Orange *et al.*, 1971; Ishizaka *et al.*, 1971; Bourne *et al.*, 1971). The effective range has been generally found to be between 1×10^{-3} M and 1×10^{-7} M of isoproterenol and 1×10^{-2} M to 1×10^{-4} M of theophylline. Discrepancies between these values and our data are probably due to differences in experimental conditions and animal species employed.

The synergism observed between theophylline and isoproterenol is consistent with a common action on PAF and histamine release via an increase in intracellular cAMP. This conclusion is supported by our observation that dibutyryl cAMP also inhibits the release of PAF and histamine in a dose–response fashion. This synergistic action and that of dibutyryl cAMP were earlier observed by investigators studying antigen-induced release of histamine and SRS-A in the primate lung (Orange *et al.*, 1971; Ishizaka *et al.*, 1971), antigen-induced histamine release in leucocytes (Lichtenstein & Margolis, 1968; Lichtenstein & de Benardo, 1971; Bourne *et al.*, 1972), and rabbit anti-rat Fab-induced histamine release in rat mastocytes (Kaliner & Austen, 1974). Our new observation of modulation by cAMP of the antigen-induced release of PAF is well in accord with published data for the other mediators of immediate hypersensitivity. However, some points merit discussion. The fact that theophylline suppresses PAF and histamine release more effectively than does isoproterenol at concentrations producing approximately equal increases in cAMP has been noted also in the histamine release from rat mastocytes (Sullivan *et al.*, 1975a, b). An explanation could be that theophylline inhibits the action of adenosine (Forments, 1975; Berne & Rubio, 1974) released from cells in response to a variety of stimuli (Bockman, Berne & Rubio, 1975; Schwabe, Ebert & Ebler, 1975; Mentzer, Rubio & Berne, 1975).

Adenosine increases the release of histamine by anti-IgE, ConA, compound 48/80, calcium ionophore A 23187 (Marquardt, Parker & Sullivan, 1978), and antigen (Okaraki & Naimal, 1972). In contrast to isoproterenol, theophylline was more inhibitory for PAF than for histamine. Such results cannot be explained by a direct effect of this substance—as well as isoproterenol—on PAF-induced platelet aggregation, as that was ruled out in our experiments. Theophylline may have an effect on the release of PAF which is independent from the inhibition of phosphodiesterase. Our results also show, in accord with others (Lichtenstein & De Benardo, 1971; Bourne *et al.*, 1972; Orange *et al.*, 1971; Ishizaka *et al.*, 1971) that isoproterenol acts on a β-receptor, as exemplified by the suppression of its inhibitory effect by propranolol, a well defined β-blocking agent. The pharmacological agents inhibited the mediator release only when present at the time of challenge with specific antigen, indicating a reversible drug-receptor interaction. This has also been observed by many other authors (Lichtenstein & Margolis, 1968; Lichtenstein & De Benardo, 1971; Bourne *et al.*, 1972; Bourne *et al.*, 1971).

In our hands, stimulation of sensitized BCC caused a fall in cellular cAMP levels, starting within seconds after antigen challenge; which reached 55% at 50 s, and persisted throughout the incubation period. This result agrees well with those reported for rat mastocytes stimulated by rabbit anti-rat Fab (Kaliner & Austen, 1974) and with the recent report of a transient increase followed by a significant fall of cAMP in basophils from a leukaemic patient stimulated with anti-IgE antibodies (Lichtenstein *et al.*, 1978). A rapid fall in adenyl cyclase was shown in pig lung challenged with antigen after passive sensitization (Sullivan & Parker, 1973). Compound 48/80 determined a remarkable and rapid decrease in cAMP during the stimulation of rat mast cells (Gillespie, 1973; Sullivan *et al.*,

1975a,b). However, Sullivan reported a rapid increase in cAMP concentration in isolated rat mast cells stimulated by ConA, and to a lesser extent, by anti-IgE antibodies (Sullivan, Parker, Kulczycki & Parker, 1976).

In our experiments, normal histamine and PAF release were observed for concentrations of isoproterenol and theophylline that counteracted the fall of cAMP, or even increased its amount. This could indicate that if the rise of cAMP concentration above given levels is indeed inhibitory, the release is not always accompanied by a fall of cAMP. However, we did not measure cAMP concentration within seconds after antigenic challenge in the presence of the drugs, threrefore missing a transient drop of cAMP. In spite of these discrepancies, our results point out the negative role of intracellular cAMP in the release of phlogistic mediators, now extended to PAF. The fall in concentration of cAMP was proportional to the antigen concentration and to the release of mediators, when no drugs were present. We never reached the point where the release would exhibit the usual bell-shaped curve as the antigen concentration increased. This indicates a lower degree of sensitization of the rabbits as compared with that previously observed in our laboratory (Benveniste *et al.*, 1976). Therefore, we could not explore a possible reversal of the cAMP level in parallel to the inhibition of the release at high doses of antigen.

The cellular population that we have studied comprised between 5 and 10% basophils (responsible for the release of histamine and PAF) 80–90% lymphomononuclear cells and 0–4% neutrophils. Our finding of a rapid fall in cAMP level can therefore only be explained by an action of the antigen on the lymphomononuclear cells, probably sensitized by the anti-peroxidase IgG produced by the rabbits with IgE antibodies during the immunization period. Antigen, theophylline and isoproterenol interfere with the cAMP system in lymphomononuclear cells (Henney, Bourne & Lichtenstein, 1972; Koopman, Gillis & David, 1972; Johnson, Blalock & Baron, 1977; Bach, 1975) and in neutrophils (Ignarro, Lint & George, 1974; Zurier, Weissman, Hoffstein, Kammerman & Tai, 1974). Other work reporting an antigen-induced fall in adenyl cyclase was also done on heterogeneous cell population (Sullivan & Parker, 1973). Nevertheless, experiments involving purification of the antibodies and of the cell populations are needed to weigh the respective role of the basophils and other cells in the fall of cAMP.

Much recent work has stressed the importance of the cAMP second messenger system in the control of phlogistic mediator release, a factor of potential importance for the management of immunopathological diseases. We now extend these observations to PAF. The cAMP system regulates the secretion of PAF, and also its effect on target cells, as shown by our present results and those of others (Henson & Oades, 1976). Platelets are capable of releasing potent vasoactive and phlogistic intermediates, and PAF is responsible for their recruitment, thereby creating an amplifying mechanism of inflammation that is instrumental in immune complex deposition (Benveniste *et al.*, 1976). PAF release can be triggered either by IgE (Siraganian & Osler, 1971) or by complement and/or neutrophil (Camussi *et al.*, 1977) dependent basophil activation. If our results in the former mechanism are confirmed in the latter, the role of the cAMP system in controlling the implication of platelets in immunopathology could be widely extended.

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