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Hemin stimulation of cAMP production in human lymphocytes

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Hemin stimulates cAMP production in human peripheral blood mononuclear cells (PBMC). The kinetics are similar to that of hormone-induced cAMP generation, namely a rapid effect followed by a desensitization phase. Several experimental findings suggest that prostaglandins do not mediate this effect. First, macrophage depleted T and B cells purified by erythrocyte-rosetting were as responsive as unfractionated PBMC to hemin. Second, indomethacin, an inhibitor of prostaglandin synthesis, and meclofenamate, a prostaglandin E_2 receptor antagonist, had no effect on hemin stimulated cAMP production. In addition, propranolol, a β-adrenergic receptor antagonist, had no effect on hemin-stimulated cAMP production. We also examined structural analogues of hemin. Among the metalloporphyrins (Fe, Ni, Co, Zn and Sn) and protoporphyrin IX tested only hemin (Fe-protoporphyrin) was active in stimulating cAMP production. No correlation was found between the ability of metalloporphyrins to stimulate cAMP production and their ability to generate H_2O_2. The data indicate that hemin stimulates cAMP production by directly affecting lymphocytes and that prostaglandins do not mediate this effect.

Prostaglandin; Metalloporphyrin; cAMP stimulation; Lymphocyte

1. INTRODUCTION

We have previously shown that hemin induced lymphocyte activation [1,2] and also found (unpublished observation) that hemin stimulates phosphotyrosine phosphatase activity in lymphocytes. Agents that increase cellular cAMP were recently found to stimulate phosphotyrosine phosphatase activity in monkey and human cell lines [3]. In this paper, we investigated the effect of hemin on cAMP production in human lymphocytes.

2. MATERIALS AND METHODS

2.1. *Cell isolation and culture*

Human PBMC were isolated from healthy volunteers by Ficoll-Hypaque density gradient centrifugation. PBMC were fractionated as follows: cells were resuspended in RPMI 1640 + 20% FCS and placed on 50 ml of a Sephadex G-10 column that was equilibrated with the same medium. After incubation at 37°C for at least 1 h cells were eluted with 50 ml of the same medium. Cells were washed, resuspended in 12 ml of RPMI 1640 and then added to 12 ml of sheep red blood cells (SRBC) and 12 ml of FCS. These were aliquoted into 8 tubes and after pelleting, allowed to sit at 4°C for at least one hour. The pellets were then layered on top of 7 ml Ficoll-Hypaque and centrifuged for 30 min. The interface contained the SRBC(−) cells and the pellet was recovered. The SRBC were lysed in sterile water for 15 s and the washed T cells were rosetted again. The twice-rosetted T cells were called SRBC(+). In some experiments, PBMC were passed over the Sephadex G-10 column twice to further macrophage-deplete the cell suspension. Flow cytometry was carried out on a Coulter Epics V flow cytometer. Cells were labeled with antibodies against CD3 (Leu 4), CD19 (Leu 12) and CD14 (Leu M3).

Hemin and the different metalloporphyrins were obtained from Porphyrin Products (Logan, UT). All other chemicals were purchased from Sigma (St. Louis, MO). Metalloporphyrins were dissolved in 1 M NaOH, neutralized with 0.5 M Tris, pH 7.5, and diluted to 500 μM stock solutions. In some experiments, hemin in the form of haem arginate (Normosang, Huhtamaki OY Pharmaceutical, Helsinki, Finland) was used.

2.2. *cAMP assay*

Human PBMC (20×10^6 cells/ml) in phosphate-buffered saline containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} and 250 μM isobutylmethylxanthine were treated with the indicated drugs for 20 min. After treatment, aliquots were boiled for 3 min and the supernatants clarified by centrifugation at $8,000 \times g$ for 2 min. Duplicate samples were assayed with a commercial kit (Amersham, Arlington Heights, IL) utilizing the cAMP binding protein.

2.3. *PGE_2 assay*

Human PBMC were incubated at 20×10^6 cells/ml in PBS for 20 min with the appropriate treatment after which cells were pelleted and samples were withdrawn for PGE_2 determination. This was done using a commercial RIA kit (Advanced Magnetics, Cambridge, MA).

2.4. *H_2O_2 assay*

The assay is based on irreversible inhibition of catalase by H_2O_2 in the presence of aminotriazole [4]. A reaction mixture containing 50 μM phosphate, pH 7.3, 20 μM aminotriazole, and 17 U bovine catalase (2 × crystallized) in 30 μl was mixed with 20 μl sample and incubated at 37°C in a shaking water bath. After 1 h, 950 μl of 82.1 mM $NaBO_3$, pH 6.8, was added and left shaking for another 5 min at 37°C. One ml of 2 N sulfuric acid was added to stop the reaction and the undestroyed perborate was titrated with 50 mM $KMnO_4$ (about 500 μl). Data are expressed as percent of destroyed perborate and a linear regression of a standard curve of known H_2O_2 was used to calculate unknowns.

Abbreviations: PBMC, peripheral blood mononuclear cells; PGE_2, prostaglandin E_2; SRBC, sheep red blood cells.

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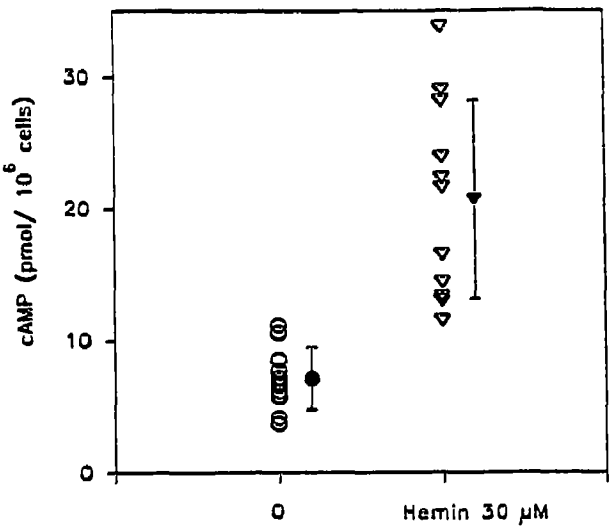

Fig. 1. Effect of hemin on cAMP levels in human lymphocytes. Human PBMC were treated for 20 min as indicated after which the samples were analyzed for cAMP levels as described in Materials and Methods. The data from 11 separate experiments are shown.

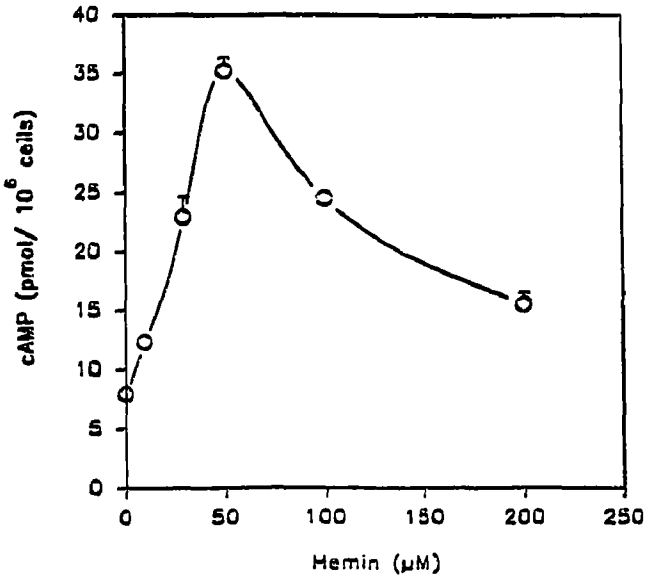

Fig. 2. Hemin-induced cAMP levels in human lymphocytes; dose effects. Human PBMC were incubated for 20 min with the indicated concentrations of hemin followed by assay of cAMP levels as described in Materials and Methods.

3. RESULTS

3.1. *Hemin stimulation of cAMP production in human PBMC*

PBMC from 11 individuals were treated separately with hemin (30 μM) for 20 min followed by cAMP determination. Untreated cells had 7.1 ± 2.3 pmol cAMP/10^6 cells (mean ± SD) and hemin-treated cells had 20.7 ± 7.5 pmol cAMP/10^6 cells ($P < 0.0001$) (Fig. 1). Hemin stimulation of cAMP production was dose dependent (Fig. 2). Hemin at 50 μM had a maximal stimulatory effect, higher concentrations of hemin were less stimulatory. Kinetics of hemin stimulation of cAMP production revealed that cAMP was maximally produced after 10 min and declined after a longer incubation period (Fig. 3). The effect of hemin on cAMP levels in human PBMC was compared with other agents known to stimulate cAMP production (Table I).

3.2. *Role of prostaglandins*

The possibility that prostaglandins mediate the stimulatory effect of hemin on cAMP production in human PBMC was investigated. PBMC contain approximately 20% monocytes which could generate prostaglandins under the experimental conditions employed. As seen in Fig. 4 indomethacin at a concentration of 30 μM, which inhibits more than 95% of the synthesis of prostaglandins in PBMC treated with mitogens [5], did not inhibit hemin stimulation of cAMP production in these cells. In addition, cAMP levels in macrophage depleted T cell fractions (SRBC(+)) and non-rosetted B cell fractions (SRBC(−)) treated with hemin were higher than in hemin-treated unfractionated PBMC (Table II).

We directly measured prostaglandin E_2 (PGE_2) production by PBMC treated with hemin. We found control PBMC to have 11.7 ± 0.3 pg $PGE_2/10^6$ cells and PBMC treated with 30μM hemin to have 16.8 ± 1.3 pg $PGE_2/10^6$ cells. This increase corresponds to approximately 20 pg PGE_2/ml. We added exogenous PGE_2 to PBMC and found no response below 100 pg PGE_2/ml. In addition meclofenamate, a receptor antagonist of PGE_2 [6], blocked the effects of exogenously added PGE_2 but had no effect on hemin-treated cells (Fig. 5).

It is interesting to note that several cell lines, including the human T cell leukemias Jurkat and Molt-4 and the erythroleukemia cell line K562 were all unresponsive to hemin, even when purified human macrophages were added (data not shown). We also investigated whether hemin mediated its effect through the β-adrenergic receptor. We found that propranolol, a β-

Table I

Comparison of hemin and cAMP generating agents

Treatment		cAMP (pmol/10^6 cells)
None		7.8
Hemin 30 μM		24.1
Isoproterenol	10 nM	15.6
	100 nM	27.8
Prostaglandin E_2	0.3 nM	43.5
	3.0 nM	77.5
	30 nM	62.6

Human PBMC were treated with the indicated concentrations of hemin, isoproterenol or prostaglandin E_2 for 20 min prior to analysis of cAMP levels as described in Materials and Methods.

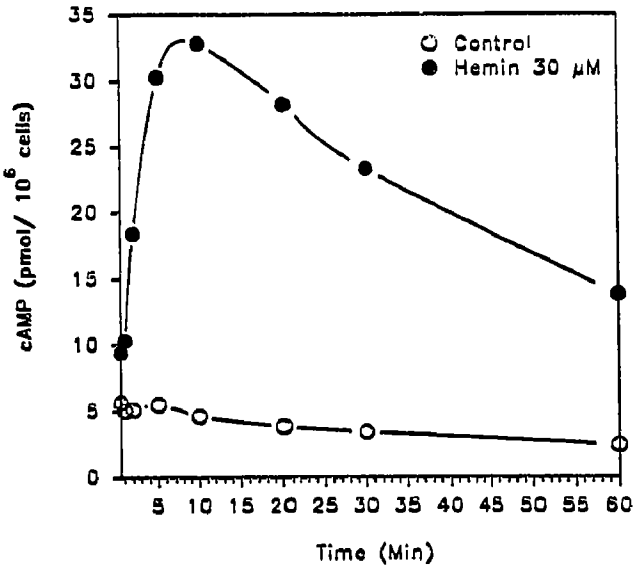

Fig. 3. Time course of hemin-induced cAMP levels in human lymphocytes. Human PBMC were incubated in the presence or absence of 30 μM hemin for the indicated times followed by assay of cAMP levels as described in Materials and Methods.

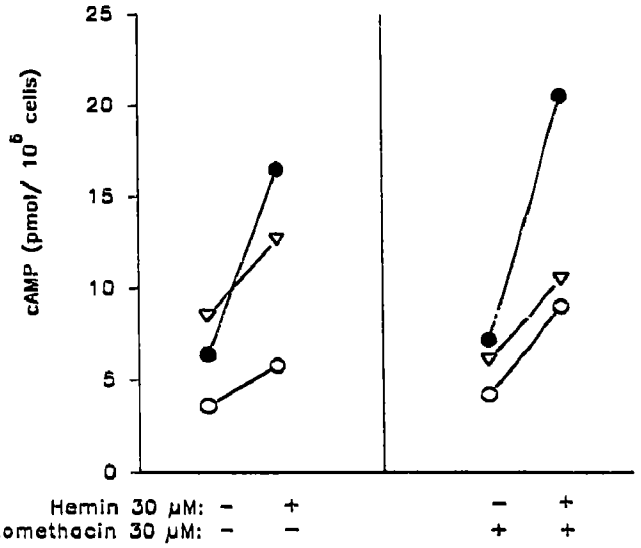

Fig. 4. Effect of indomethacin on hemin-induced cAMP levels. Human PBMC were treated with the indicated combinations of hemin and indomethacin for 20 min followed by assay of cAMP levels as described in Materials and Methods. The data are from 3 separate experiments.

adrenergic receptor antagonist, blocked the ability of epinephrine but not that of hemin to induce cAMP production (Fig. 5).

3.3. *Effect of hemin analogues*

We have evaluated the effect of a variety of metalloporphyrins for their capacity to stimulate cAMP production in PBMC. Among the Fe-, Ni-, Co-, Zn- and Sn-protoporphyrins, only Fe-protoporphyrin (hemin) showed a marked stimulatory activity. Ni-protoporphyrin was slightly stimulatory and Sn-protoporphyrin was slightly inhibitory. Co- and Zn-protoporphyrins and protoporphyrin IX had no effect (Fig. 6).

Hemin may mediate its effects through an oxidative process. We measured the generation of H_2O_2 catalyzed by the different metalloporphyrins using the aminotriazole-catalase inhibition assay that we have previously developed [4]. As seen in Table III, the hierarchy of potency of the different metalloporphyrins to generate H_2O_2 was as follows:

Fe = Co > Zn > Sn > Ni > protoporphyrin IX

No correlation was found between the ability of metalloporphyrins to stimulate cAMP production and their ability to generate H_2O_2.

Table II

Effect of hemin on fractionated PBMC

Treatment	cAMP (pmol/10^6 cells)		
	PBMC	SRBC(+)	SRBC(−)
None	3.0	5.8	0.4
Hemin 30 μM	7.2	39.6	21.5
Isoproterenol 100 nM	20.1	32.1	17.0
	Flow cytometry (% positive)		
Marker	PBMC	SRBC(+)	SRBC(−)
CD3 (T cells)	74.6	77.0	6.1
CD19 (B cells)	10.2	3.8	17.0
CD14 (monocytes)	18.3	0.3	3.1

Human PBMC were separated into the three fractions and then incubated for 20 min with the indicated treatment prior to measurement of cAMP. The antibodies used for flow cytometry were directed against Leu 4 (CD3), Leu 12 (CD19), and Leu M3 (CD14). All procedures are described in Materials and Methods.

4. DISCUSSION

Here we have reported the stimulation of cAMP production in human peripheral lymphocytes by hemin. The kinetics are similar to that of hormone-induced cAMP generation, namely a rapid effect followed by a desensitization phase. Prostaglandins, products of macrophages which are known to stimulate cAMP production, do not seem to mediate the stimulatory effect of hemin. This is based on the findings that indomethacin, at concentrations which inhibit prostaglandin synthesis, and meclofenamate, a PGE_2 receptor antagonist, did not abrogate the effect of hemin. Although hemin-treated PBMC generated a small amount of PGE_2, this amount, when added exogenously was insufficient to induce cAMP in PBMC. Moreover, hemin stimulated cAMP production in purified human T cells depleted of

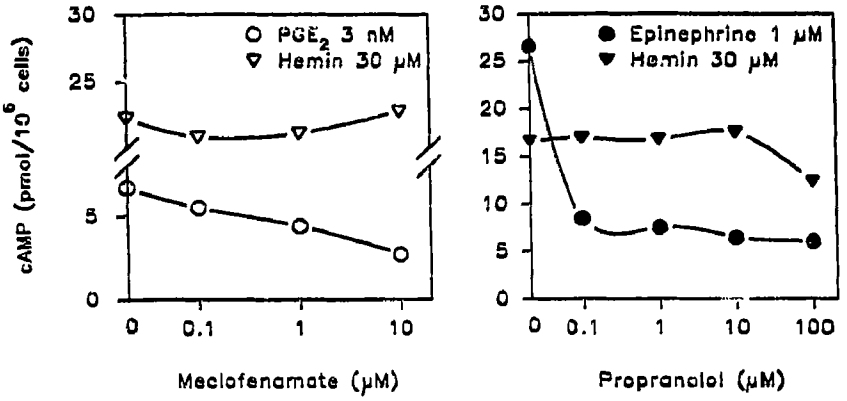

Fig. 5. Effect of meclofenamate and propranolol on hemin-induced cAMP levels. Human PBMC were treated with the indicated drug for 20 min in the presence of various concentrations of meclofenamate or propranolol followed by measurement of cAMP levels as described in Materials and Methods.

macrophages and also in the presence of indomethacin. The ability of macrophage depleted, erythrocyte-rosetted positive and negative cells to respond to hemin implies that both T and B cells are responding in PBMC. It is interesting to note that the T cell leukemic cell lines Jurkat and Molt-4 and the erythroleukemia K562 were all unresponsive to hemin. Hemin-induced increased levels of cAMP in human PBMC does not seem to result from inhibition of cAMP phosphodiesterase. This conclusion is based on our findings that hemin stimulated cAMP production in the presence of the cAMP phosphodiesterase inhibitor, isobutylmethylxanthine. In addition, the β-adrenergic receptor does not seem to be the target of hemin as propranolol, a β-adrenergic receptor antagonist, blocked epinephrine- but not hemin-stimulated cAMP production.

We also considered the possibility that the stimulatory effect of hemin is related to its oxidative properties. We could not find any correlation between the oxidative properties of a variety of metalloporphyrins as determined by H_2O_2 production and their ability to stimulate cAMP production. Hemin is among a class of newly identified lymphocyte mitogens termed the ferro-mitogens [7]. It is of interest to note that cAMP, a product of hemin stimulation, has been shown to negatively regulate a variety of lymphocyte responses [8–11]. Hemin is known to induce globin gene transcription in erythroleukemia cell lines and to induce differentiation of these cells. The possible role of hemin-induced cAMP in these processes is open to further investigation.

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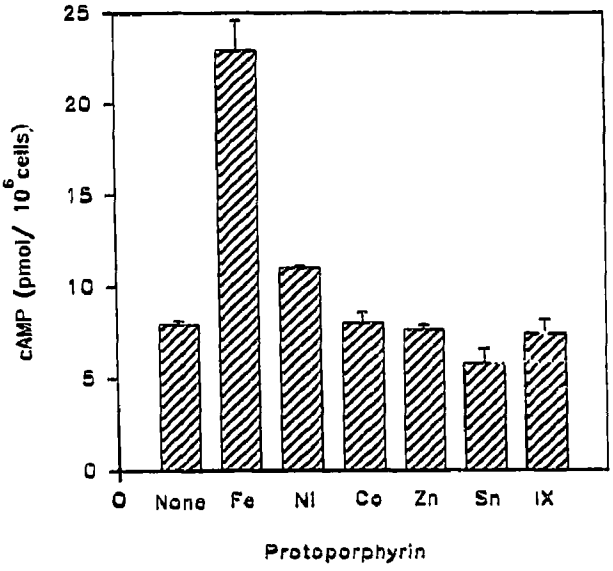

Fig. 6. Effect of hemin analogues on cAMP levels in human lymphocytes. Human PBMC were incubated for 20 min with 30 μM of the indicated hemin analogue and subsequently assayed for cAMP levels as described in Materials and Methods. IX indicates protoporphyrin IX, without a central metal.

Table III

Generation of H_2O_2 by metalloporphyrins

Protoporphyrin (30 μM)	H_2O_2 generated (μM)
Fe	440
Ni	37
Co	440
Zn	266
Sn	90
IX	0

Each metalloporphyrin was incubated for 1 h at 37°C in the presence of aminotriazole and catalase as described in Materials and Methods. IX denotes protoporphyrin IX.

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