CHANGES IN INTRACELLULAR PROSTAGLANDIN CONTENT DURING ACTIVATION OF LYMPHOCYTES BY PHYTOHAEMAGGLUTININ

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1. Introduction

Prostaglandins have been implicated in many cellular control processes and the differential roles of the E series and F_α series prostaglandins in their effects on DNA synthesis and cell division have been well studied [1–4]. Both affect the intracellular pools of cyclic nucleotides. PGE_1 markedly increases the accumulation of cAMP and stimulates adenyl cyclase activity in rat lymphnode lymphocytes [5], and increases cAMP levels in tonsillar lymphocytes [6] and in transformed 3T3 cells [7]. $PGF_{2\alpha}$ has been reported to cause cGMP accumulation in 3T3 cells [1].

Several lines of evidence have suggested that synthesis of endogenous prostaglandins may play a part in lymphocyte stimulation. Enhanced accumulation of PGE has been found [8] in cultures of mouse spleen cells stimulated with endotoxin or concanavalin for 1 or 2 days and a very large increase in splenic $PGF_{2\alpha}$ has been reported within minutes of injection of sheep erythrocytes into sensitized mice [9,10]. Aspirin, an inhibitor of prostaglandin synthesis, inhibits mitogen- and antigen-stimulated [^{3}H]thymidine incorporation by human blood lymphocytes [11], while another inhibitor of prostaglandin synthesis, indomethacin, inhibits DNA synthesis in cultured mouse fibroblasts [1]. Changes in endogenous prostaglandin production could also account for some of the various changes in the intracellular pools of cAMP and cGMP reported to occur soon after the addition of mitogen to lymphocytes [12–16].

We have therefore determined the changes in intracellular PGE_2 and $PGF_{2\alpha}$ during the period immediately following the addition of the mitogen PHA to lymphocytes. We have found that there is a rapid but transient increase in intracellular PGE_2 that is maximal 30–60 min after the addition of mitogenic concentrations of PHA. There was no corresponding early increase in $PGF_{2\alpha}$ at any PHA concentration tested, although $PGF_{2\alpha}$ levels did increase later in culture. Inhibitors of prostaglandin synthesis prevented the early increase in PGE_2 but did not greatly affect other aspects of lymphocyte activation by the mitogen.

Abbreviations: PHA, phytohaemagglutinin; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$

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2. Materials and methods

2.1. *Preparation and incubation of lymphocyte cultures*

Lymphocytes were purified from 1–2 l batches of pig blood by a method detailed in [17]. This method includes defibrination, which removes all platelets; sedimentation with dextran, to remove most erythrocytes; passage of the cells through a column of cotton wool at 37°C to remove phagocytic cells; and separation of lymphocytes from residual erythrocytes by centrifugation on ficoll–hypaque step gradients. The cells obtained by this procedure were at least 99% lymphocytes by morphological criteria. They were incubated at 37°C at 2×10^6 cells/ml in Eagle's minimal essential medium containing 15% autologous serum.

2.2. *Extraction and assay of prostaglandins*

The cells were collected by centrifugation, washed and resuspended in 0.5 ml 0.15 M NaCl and finally broken by freezing and thawing quickly in a mixture of dry ice and ethanol. Prostaglandins were extracted in 16 vol. ethanol : ether (3:1) [18] and after centrifugation the upper layer was removed and dried in a stream of N_2. The extract was dissolved in phosphate-buffered saline and the prostaglandin content was determined by a modification of the radioimmunoassay method in [19]. Three determinations at different concentrations of extract, each in triplicate, were made. Each value given is the mean ± SE. The standard curve was obtained using known concentrations of the relevant prostaglandin which had been dissolved in ethanol : ether (3:1), dried under a stream of N_2 and resuspended in buffer. The percentage extraction of prostaglandins was 75–80%.

2.3. *Measurement of [^{14}C]leucine and [^{3}H]thymidine incorporation and [^{3}H]uridine uptake*

Triplicate 1 ml cultures (2×10^6 cells/ml) were preincubated for 1 h with differing concentrations of inhibitors of prostaglandin synthesis before the addition of PHA (15 μg/ml). [^{14}C]Leucine and [^{3}H]thymidine were added to a final 1 μCi/ml, 4 h and 48 h, respectively, after the addition of PHA and their incorporation determined after incubation for a further 2 h. The cells were then harvested, washed with 0.15 M NaCl and the trichloroacetic acid-insoluble material collected on a glass fibre filter. The radioactivity incorporated was determined using a Beckman scintillation counter.

To determine the rate of [^{3}H]uridine uptake cultures were incubated with 5 μCi/ml [^{3}H]uridine for 1 h at 37°C. Uptake of the isotope was approximately linear over this period. The cells were then washed 3 times with 0.15 M NaCl, lysed by addition of 1 ml cold 10% trichloracetic acid, and the acid-soluble radioactivity in duplicate 0.25 ml samples determined.

2.4. *Materials*

PHA-P was obtained from Difco Laboratories, Detroit, MI. Indomethacin, phenylbutazone and aspirin were purchased from Sigma Chemical Co., St Louis, MO. Ethanol and diethyl ether were of Analar grade. [*methyl*-^{3}H]Thymidine (5 Ci/mmol), L-[U-^{14}C]leucine (354 mCi/mmol), [5-^{3}H]uridine (29 Ci/mmol), [5,6,8,11,12,14,15(*n*)-^{3}H]PGE_2 and [5,6,8,11,12,14,15(*n*)-^{3}H]$PGF_{2\alpha}$ (120–170 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks.

3. Results

3.1. *Changes in intracellular concentrations of PGE_2*

The changes in intracellular concentrations of PGE_2 during the first 2 h after the addition of PHA to unstimulated lymphocytes are shown in fig.1. There was a distinct rise after 10 min and after 30–60 min there was a 7-fold increase over basal levels. In 6 separate experiments the maximum increase was 4–7-fold and always occurred from 30–60 min. Lymphocytes incubated with PHA for 6 h or 20 h contained 1.5–2-fold more PGE_2 than unstimulated cells.

Indomethacin, which is reported to inhibit prostaglandin synthesis [20], was tested to determine whether it affected this increase in intracellular levels of PGE_2. In preliminary experiments with PHA-stimulated lymphocytes incubation for 1 h with 20 μM indomethacin was found to substantially reduce intracellular PGE_2. In the experiment shown in fig.2 the cells were preincubated for 1 h with 20 μM indomethacin before the addition of PHA. No significant increase in PGE_2 was seen.

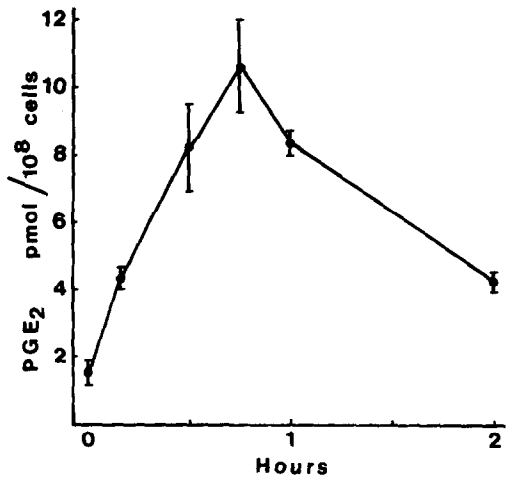

Fig.1. Changes in intracellular concentrations of PGE_2 during the first 2 h after addition of PHA (15 μg/ml). The concentration of PGE_2 after 20 h was 2.67 ± 0.25 pmol/10^8 cells.

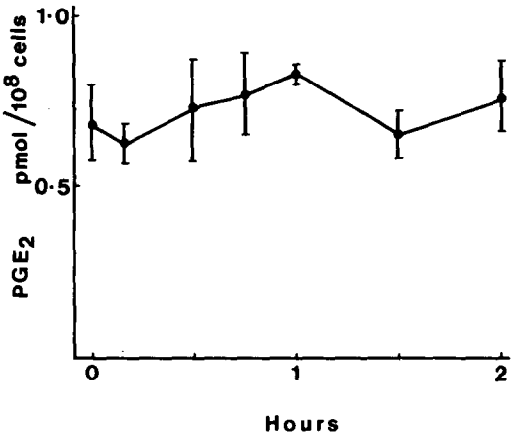

Fig.2. Effect of indomethacin on intracellular PGE_2 concentrations. Cells were preincubated for 1 h with 20 μM indomethacin before the addition of PHA (15 μg/ml) at time 0. Intracellular PGE_2 concentration after 20 h in the presence of PHA was 1.03 ± 0.096 pmol/10^8 cells.

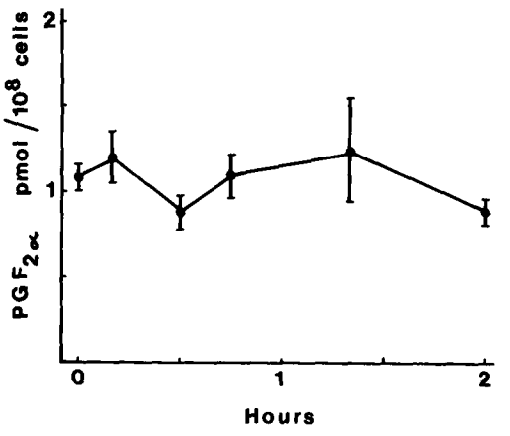

Fig.3. Changes in intracellular concentrations of $PGF_{2\alpha}$ in the first 2 h after addition of PHA (15 μg/ml). After 20 h the intracellular $PGF_{2\alpha}$ content was 7.2 ± 0.28 pmol/10^8 cells.

When the effect of different concentrations of PHA on the increase in intracellular PGE_2 content after incubation with PHA for 30 min was studied, a direct relationship was found between intracellular PGE_2 content and PHA $\leqslant$ 15 μg PHA/ml. Addition of further PHA did not further increase the level of intracellular PGE_2. At PHA $<$ 1.5 μg/ml there was little or no increase in intracellular PGE_2 content over the level found in unstimulated lymphocytes. Three such experiments gave essentially similar results.

3.2. *Changes in intracellular concentrations of $PGF_{2\alpha}$*

There was no early increase in intracellular levels of $PGF_{2\alpha}$ comparable to that found to PGE_2 (fig.3). The intracellular $PGF_{2\alpha}$ content was not statistically different from the unstimulated level at any time within 2 h of the addition of PHA. After incubation for 20 h with PHA there was an increase which varied 2–7-fold over that found in unstimulated lymphocytes in 5 separate experiments.

No concentration of PHA in the range tested produced an early increase in intracellular $PGF_{2\alpha}$ concentration (fig.4). At 3.75 μg/ml and 7.5 μg/ml PHA the amount of $PGF_{2\alpha}$ present in the cells was slightly reduced. Three such experiments gave essentially similar results.

3.3. *Effect of inhibitors of prostaglandin synthesis on lymphocyte activation*

Table 1 shows the effect of 3 drugs reported to inhibit prostaglandin synthesis on [^{3}H]thymidine incorporation into DNA by PHA-stimulated lymphocytes. At none of the concentrations tested was there complete inhibition of [^{3}H]thymidine incorporation.

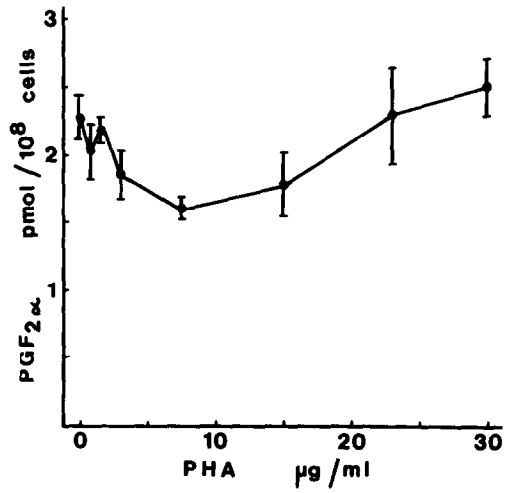

Fig.4. Changes in intracellular $PGF_{2\alpha}$ concentrations in cells incubated with different concentrations of PHA for 30 min. After 20 h in the presence of PHA (15 μg/ml) the intracellular $PGF_{2\alpha}$ content was 4.28 ± 0.16 pmol/10^8 cells.

Table 1
Effect of inhibitors of prostaglandin synthesis on [^{3}H]thymidine incorporation by PHA-stimulated cells

Inhibitor	cpm × 10^4 ± SD	% Inhib.
Control – PHA	0.12 ± 0.01	–
Control + PHA	3.51 ± 0.25	–
Indomethacin		
1 μM	3.09 ± 0.25	12
5 μM	2.88 ± 0.21	18
20 μM	2.71 ± 0.15	23
Phenylbutazone		
5 μM	3.01 ± 0.21	14
10 μM	2.91 ± 0.18	17
50 μM	2.88 ± 0.22	18
Aspirin		
1 μM	3.28 ± 0.27	6
5 μM	2.94 ± 0.22	16
20 μM	2.67 ± 0.21	24
50 μM	1.95 ± 0.17	44

Each value is the average of 3 determinations. Each culture contained 15 μg/ml PHA. The inhibitors were dissolved in ethanol : Eagles medium, 1:10 (v/v). The control cultures contained equivalent volumes of ethanol : Eagles. Three such experiments gave essentially similar results

With 20 μM indomethacin, which we have shown completely inhibits the early rise in intracellular PGE_2 content (fig.2), there was only 23% inhibition of [^{3}H]thymidine incorporation. Similar inhibition was seen with phenylbutazone. The greatest inhibition (44%) was shown by 50 μM aspirin.

Figure 5 shows the effect of indomethacin on [^{3}H]uridine uptake in the first hour after the addition of PHA and on [^{14}C]leucine incorporation into protein after 6 h stimulation with PHA. In neither case was there any significant effect of indomethacin on the mitogen-dependent increases seen.

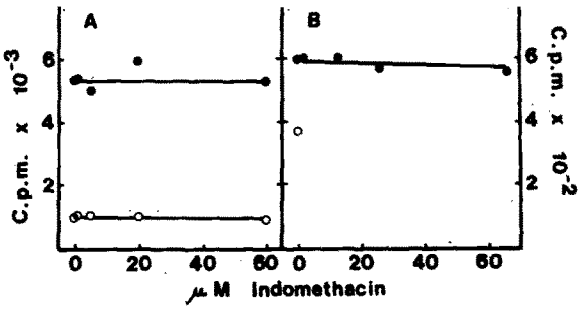

Fig.5. Effects of indomethacin of (A) [^{3}H]uridine uptake into the acid-soluble fraction by lymphocytes incubated with or without PHA for 1 h and (B) [^{14}C]leucine incorporation into protein by lymphocytes incubated with PHA for 6 h. Cells were preincubated with indomethacin for 1 h before the addition of PHA at time 0. (•) Cultures with PHA. (○) Cultures without PHA.

4. Discussion

The large early transient increases in intracellular PGE_2 observed in lymphocytes incubated with PHA in this study have not been reported previously. However, long term changes in medium concentrations of PGE_2 compatible with the increase we observed after 20 h in the presence of PHA have been seen in both serum-stimulated fibroblasts and mitogen-stimulated mouse spleen cells [7,8]. We did not find any early transient increase in $F_{2\alpha}$ as has been reported in whole spleens in sensitized mice injected with antigen [9,10], but did observe a later and rather variable rise as has been seen by others in serum-stimulated fibroblasts [21]. The transient rise in PGE_2 correlates with, and may be the cause of, the increase in cAMP seen by some [13,15,22] but not all [12,14] workers. However, the role of prostaglandins could be unrelated to their effect on cyclic nucleotides as endogenously produced prostaglandins may function as regulators of cell division per se [23].

Indomethacin and aspirin, two inhibitors of prostaglandin synthesis, have been reported to inhibit stimulation of DNA synthesis in serum-stimulated fibroblasts and mitogen-stimulated lymphocytes, respectively [1,11]. However, at concentrations reported to inhibit prostaglandin synthetase completely both these compounds and phenylbutazone, another prostaglandin synthesis inhibitor [1,23,24], only partly inhibited PHA-stimulated [^{3}H]thymidine incorporation. It is difficult to be certain that the inhibition of prostaglandin synthesis is fully maintained over the 48 h culture period required for measurement of DNA synthesis or that the inhibitions seen in these experiments are not due to secondary effects of the drugs. However, the concentration of indomethacin which abolishes the early transient rise in PGE_2 does not affect the simultaneous increase in uridine uptake

or early increase in protein synthesis seen a few hours later. We have, therefore, been unable to conclude that the early transient rise in PGE_2 observed is necessary for the mitogenic response to PHA. It may, however, be related to other T-cell functions induced by mitogens such as acquisition of suppressor cell function [25].

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