

Evidence to suggest that the phosphodiesterase 4 isoenzyme is present and involved in the proliferation of umbilical cord blood mononuclear cells

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Summary

Background The type 4 phosphodiesterase (PDE) isoenzyme is the main isoenzyme of PDE involved in the control of adult mononuclear cell proliferation.

Objective To establish whether PDE isoenzymes are present in umbilical cord blood mononuclear cells by the use of selective PDE inhibitors, and to identify which PDE isoenzymes are involved in controlling the proliferation of cord blood mononuclear cells.

Methods Cord blood was obtained from normal deliveries and mononuclear cells isolated as described previously [1] with some modifications. Mononuclear cells were then stimulated to proliferate with phytohaemagglutinin (PHA) (2 µg/mL) in the presence of selective PDE inhibitors. Proliferation was measured by [³H]-thymidine incorporation.

Results The type 4 PDE inhibitors (CDP840, rolipram and RO 20-1724), and the mixed PDE3/4 inhibitor, zardaverine, produced a concentration-related inhibition of PHA-stimulated cord blood mononuclear cell proliferation ($P < 0.05$, ANOVA). The non-selective PDE inhibitor, theophylline, also produced a concentration-related inhibition of proliferation ($P < 0.05$, ANOVA). In contrast, the PDE1 inhibitor, vinpocetine, the PDE3 inhibitor, siguazodan, and the PDE5 inhibitor, zaprinast, were unable to inhibit cord blood mononuclear cell proliferation.

Conclusion PDE4 is present in umbilical cord mononuclear cells and is involved in the control of cord blood mononuclear cell proliferation.

Keywords: isoenzyme selective PDE inhibitors, cord blood mononuclear cells

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Introduction

Activated T-lymphocytes are thought to orchestrate the allergic inflammatory response seen in atopic diseases such as bronchial asthma and atopic dermatitis [2].

Elevation of intracellular levels of the cyclic nucleotide, cAMP, inhibits a variety of lymphocyte functions, including lymphocyte blastogenesis [3,4], proliferation [5] and activation [6–12]. One mechanism by which cAMP may be elevated within cells is by inhibition of the PDE enzyme. The PDE enzyme exists in at least seven different isoenzyme

forms [13] which are identified by substrate specificity, sensitivity to endogenous co-factors and sensitivity to different inhibitors [14]. The predominant PDE isoenzyme in most inflammatory cells is PDE 4 [15]. However, the type 3, 4 and 7 PDE isoenzymes have been characterized [4,16,17,18] and are known to control cAMP breakdown in adult human T-lymphocytes [10].

Abnormalities of immune cell function have been identified in atopic disease. For example, one study demonstrated an excessive histamine release by atopic basophils [19], and another demonstrated hyper IgE synthesis by mononuclear cells from atopic dermatitis patients [20]. These abnormalities may be due to defects in the cyclic nucleotide regulatory system. Indeed, there is evidence to suggest that cAMP

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hydrolytic activity is increased in mononuclear leukocytes from patients with atopic disease [21], which has been attributed to an elevated cAMP specific PDE activity [19–21]. Moreover, the more active cAMP–PDE isoform in atopic leukocytes has been demonstrated to have a greater sensitivity to inhibition by the type 4 PDE inhibitor, RO 20-1724, than the PDE in normal leukocytes [22]. In addition, it has previously been demonstrated that mononuclear cells from patients with atopic dermatitis are more sensitive to the anti-proliferative effects of PDE4 inhibitors than are mononuclear cells from patients with no history of atopic disease [1].

Our ultimate aim is to examine whether there are differences in the activity of PDEs at birth between atopic and non-atopic patients, or whether this altered sensitivity to PDE4 inhibitors is something which develops later in life.

The aim of the present study was to identify, by the use of selective PDE isoenzyme inhibitors, whether PDE isoenzymes are present in cord blood mononuclear cells and if so, whether they are involved in controlling the proliferation of these cells.

Methods

This study was approved by the Ethics Committee of King's College Hospital.

Optimization of cell proliferation assay

Venous cord blood (approximately 10 mL) was obtained from normal deliveries or elective caesarean sections and collected in tubes coated with either the anti-coagulant ethylenediamine tetracetate (EDTA) or heparin. The samples were stored in the fridge until transport to the laboratory. Samples were processed within 6 h of venesection. The blood was diluted with 0.9% NaCl (10 mL blood: 15 mL NaCl), added to a leucosep tube (Poly Labo, Paris, France) containing 15 mL Histopaque (Sigma Diagnostics, Poole, UK) (density = 1.077 ± 0.001 g/mL) beneath a porous barrier and centrifuged for 10 min at 1100 *g* (20°C). The plasma was then aspirated and mononuclear cells were collected with a Pasteur pipette. Sterile NaCl (5 mL of 0.9%) was added to the recovered mononuclear cell fraction and the fraction centrifuged for 10 min at 1100 *g* (0°C). The supernatant fluid was then aspirated and the cell pellet re-suspended in ammonium chloride (0.83% dissolved in distilled water) to lyse red blood cells. Samples were then left in the fridge for 15 min prior to being centrifuged for a further 10 min at 1100 *g* (0°C). (Parallel experiments were performed in which red blood cells were not lysed for comparison). The supernatant fluid was then aspirated, the cells resuspended in 5 mL sterile NaCl and centrifuged for a further 10 min. Following removal of the supernatant fluid, cells were resuspended in Spinners

modified essential medium (SMEM) containing L-glutamine (2 mmol/L), non-essential amino acids (1% v/v), sodium pyruvate (1 mmol/L), penicillin (100 U/mL), streptomycin (100 µg/mL) and HEPES (20 mmol/L). Total cell counts were performed using an improved Neubauer haemocytometer and cell viability was assessed by trypan blue exclusion. The volume of cells was then adjusted to 10^6 cells/mL in culture medium with 10% Ultrosor (serum substitute).

Concentration response curves for PHA

Mononuclear cells (10^5) were seeded in 96-well plates in a volume of 100 µL, and 100 µL PHA were added to each well at final concentrations of 0–20 µg/mL. The cells were then incubated at 37 °C in a 95% air, 5% CO₂ atmosphere.

Twenty-four hours later, [³H]-thymidine (0.1 µCi) was added to each well diluted in culture medium and the cells were incubated for a further 24 h period. Cells were then harvested onto glass fibre filters using a cell harvester (ICN Flow, Buckinghamshire, UK) and counted in a scintillation counter.

Studies with PDE inhibitors

Cord blood was collected in EDTA anti-coagulated tubes. Mononuclear cells were isolated as described above and red blood cells were lysed. Mononuclear cells (10^5) were added to each well of a 96-well plate in a volume of 100 µL, and 50 µL PHA were added to each well at a final concentration of 2 µg/mL. This concentration of PHA was selected as it produced a sub-maximal stimulation of proliferation. PDE inhibitor (50 µL) or vehicle was then added to each well. Control wells received 50 µL culture medium and 50 µL of the vehicle, dimethylsulphoxide (DMSO), which was at a final concentration in each well of no greater than 0.1%. This was the final concentration of DMSO in cells that were treated with the highest concentration, 10 µmol/L, of PDE inhibitor. This concentration of DMSO was found to have no significant effect on cell proliferation. Cells were then incubated as described above and proliferation quantified by [³H]-thymidine incorporation.

Statistical tests

Each drug was examined on four to six cord blood samples, and each concentration of PDE inhibitor was examined in triplicate. IC₅₀ values were calculated together with 95% confidence limits. Data are expressed as mean $\pm$ SE mean. Concentration response curves were analysed by ANOVA, and drug-treated cells were compared with vehicle-treated cells by Dunnett's test.

Materials

SMEM, L-glutamine, non-essential amino acids (10 $\times$),

sodium pyruvate, penicillin streptomycin (Gibco), HEPES, DMSO, Histopaque, trypan blue and chromotrope 2R Erlich's Haematoxylin were obtained from Sigma Diagnostics, Poole, UK. Ultrosor was obtained from Jones Chromatography, Hengoed, Mid-Glamorgan, UK. PHA and leucosept tubes were obtained from Poly Labo, Paris, France. [^3H]-thymidine was purchased from Amersham International, Little Chalfont, Buckinghamshire, UK. PDE inhibitors were obtained as follows: vinpocetine (Gedeon Richter Ltd, Budapest, Hungary), siguazodan (SmithKline Beecham Pharmaceuticals, King of Prussia, PA, USA), rolipram (Schering AG, Berlin, Germany), RO 20-1724 (Calbiochem, Nottingham, UK), CDP840 (Celltech Ltd, Slough, UK), zardaverine (Forschungslaboratorien Byk Gulden, Germany) and zaprinast (Rhone-Poulenc Rorer, Dagenham, UK).

Results

PHA produced a bell-shaped concentration-related stimulation of proliferation of mononuclear cells isolated from both

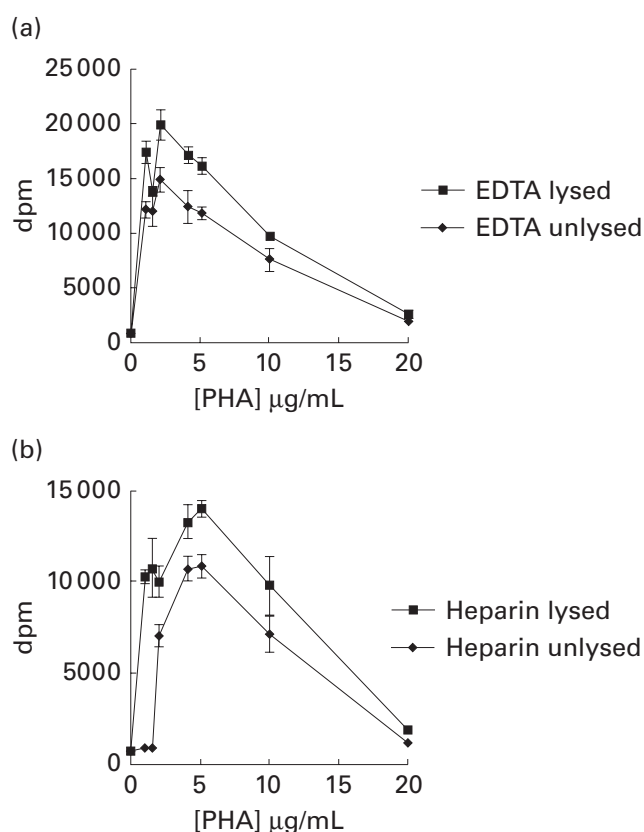


Fig. 1. Effect of PHA (0–20 µg/mL) on the proliferation of umbilical cord blood mononuclear cells with red blood cells lysed or unlysed and blood collected in (a) EDTA anti-coagulated blood or (b) heparin anti-coagulated blood. Values are mean $\pm$ SE mean ($n = 4$ per group).

heparin and EDTA anti-coagulated blood. Mononuclear cells isolated from EDTA anti-coagulated blood, however, proliferated to a greater extent than those from heparin anti-coagulated blood. Lysis of red blood cells resulted in an increased proliferative response of mononuclear cells to PHA. A concentration of 2 µg/mL produced a sub-maximal stimulation of proliferation and was selected for experiments with PDE inhibitors (Fig. 1).

The type 1 PDE isoenzyme inhibitor, vinpocetine (Fig. 2), the type 3 PDE inhibitor, siguazodan (Fig. 3) and the type 5 PDE inhibitor, zaprinast (Fig. 4) had no effect on PHA-stimulated cord blood mononuclear cell proliferation.

In contrast, the PDE4 inhibitors (rolipram, RO 20-1724 and CDP840) produced a concentration-related inhibition of proliferation (Figs 5, 6 and 7, respectively) ($P < 0.05$, ANOVA). This achieved statistical significance at a concentration of 1 µmol/L for CDP840 and at 3 µmol/L for rolipram and RO 20-1724 ($P < 0.05$). The IC_{50} value for CDP840 was 1.45 µmol/L (95% confidence limits (CL): 0.475–4.400, $n = 5$), for RO 20-1724, 3.53 µmol/L: 0.402–31 µmol/L, $n = 5$ and for rolipram > 10 µmol/L. The mixed PDE3/4 inhibitor, zardaverine, also produced a concentration-related inhibition of PHA-stimulated proliferation ($P < 0.05$, ANOVA) with an IC_{50} value of 1.18 µmol/L: 0.485–2.87 µmol/L, $n = 5$ (Fig. 8).

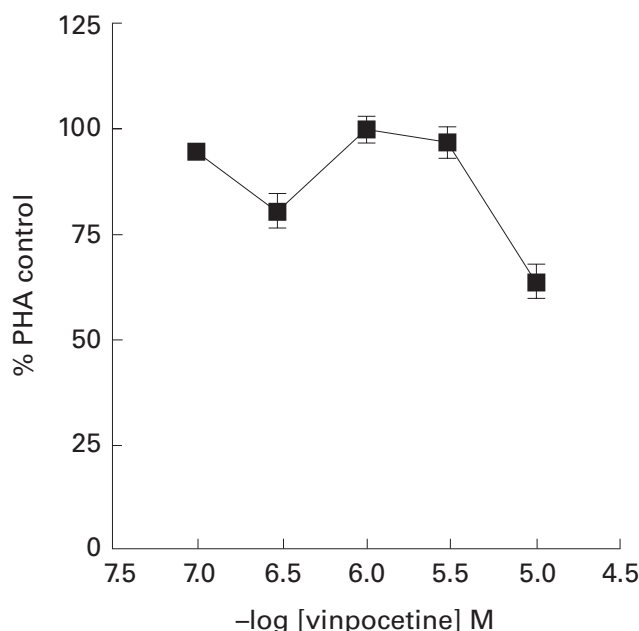


Fig. 2. Effect of vinpocetine on the PHA (2 µg/mL) stimulated proliferation of umbilical cord blood mononuclear cells. Data are expressed as percentage of the values obtained for cells stimulated with PHA in the absence of drug. Values are mean $\pm$ SE mean ($n = 5$).

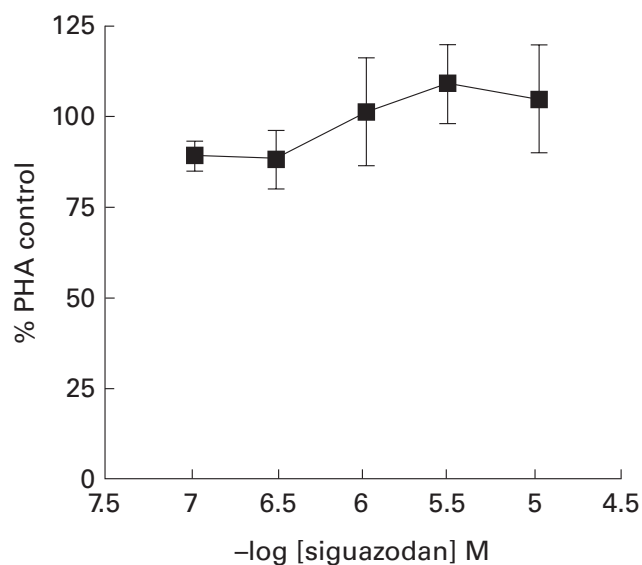


Fig. 3. Effect of siguazodan on the PHA ($2\mu\text{g/mL}$) stimulated proliferation of umbilical cord blood mononuclear cells stimulated with PHA in the absence of drug. Values are mean $\pm$ SE mean ($n=5$).

The non-selective PDE inhibitor, theophylline, produced a concentration-related inhibition of proliferation with an IC_{50} value of $396\mu\text{mol/L}$: $122\text{--}1291\mu\text{mol/L}$, $n=5$ (Fig. 9). This inhibition achieved statistical significance at a concentration of $300\mu\text{mol/L}$.

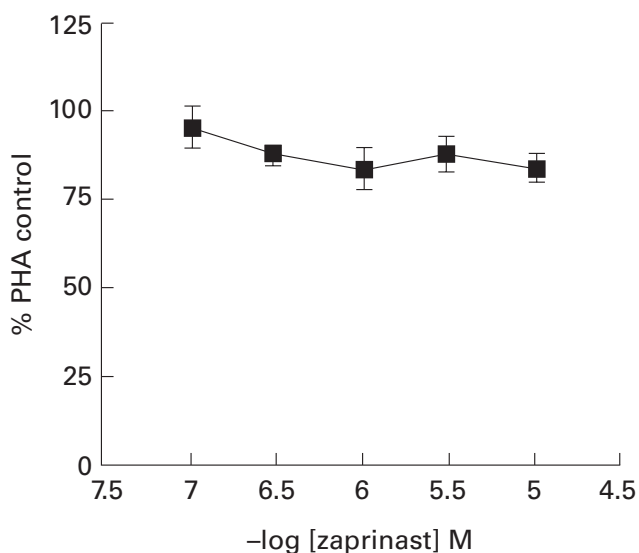


Fig. 4. Effect of zaprinast on the PHA ($2\mu\text{g/mL}$) stimulated proliferation of umbilical cord blood mononuclear cells. Data are expressed as percentage of the values obtained for cells stimulated with PHA in the absence of drug. Values are mean $\pm$ SE mean ($n=5$).

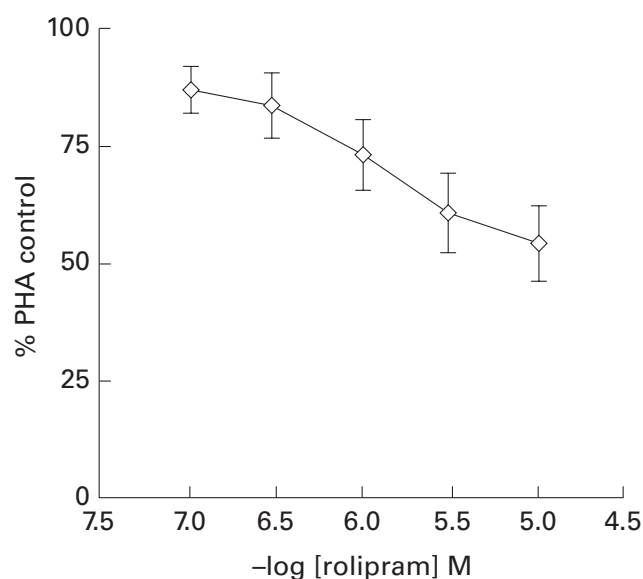


Fig. 5. Effect of rolipram on the PHA ($2\mu\text{g/mL}$) stimulated proliferation of umbilical cord blood mononuclear cells. Data are expressed as percentage of the values obtained for cells stimulated with PHA in the absence of drug. Values are mean $\pm$ SE mean ($n=5$).

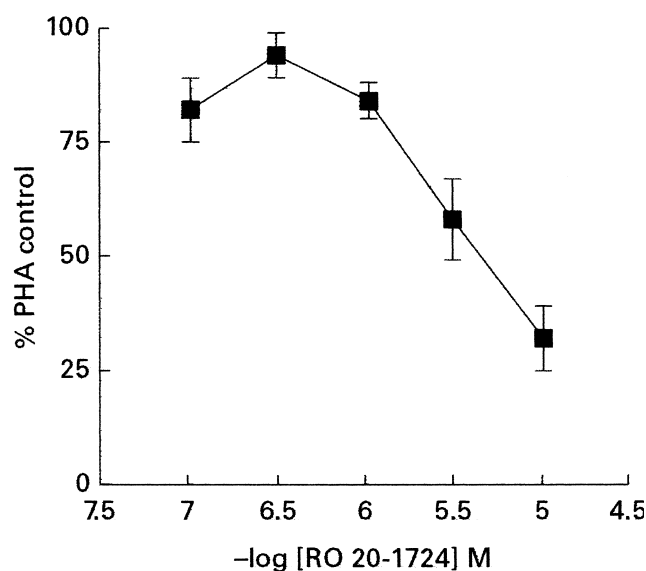


Fig. 6. Effect of RO 20-1724 on the PHA ($2\mu\text{g/mL}$) stimulated proliferation of umbilical cord blood mononuclear cells. Data are expressed as percentage of the values obtained for cells stimulated with PHA in the absence of drug. Values are mean $\pm$ SE mean ($n=5$).

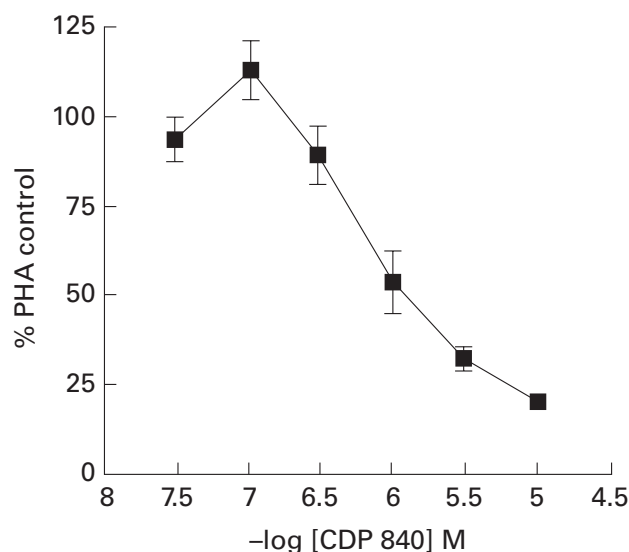


Fig. 7. Effect of CDP840 on the PHA ($2\text{ }\mu\text{g/mL}$) stimulated proliferation of umbilical cord blood mononuclear cells. Data are expressed as percentage of the values obtained for cells stimulated with PHA in the absence of drug. Values are mean $\pm$ SE mean ($n=5$).

Discussion

An increase in cAMP levels via the inhibition of PDE has been shown to inhibit the activation of a variety of cells thought to be involved in the inflammatory response,

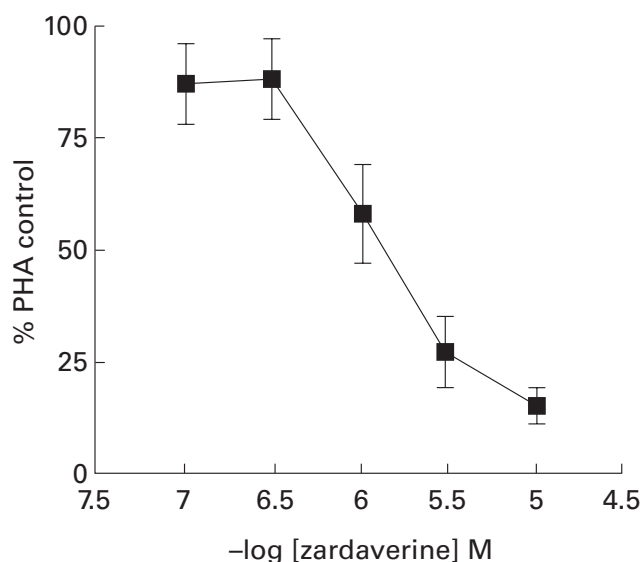


Fig. 8. Effect of zardaverine on the PHA ($2\text{ }\mu\text{g/mL}$) stimulated proliferation of umbilical cord blood mononuclear cells. Data are expressed as percentage of the values obtained for cells stimulated with PHA in the absence of drug. Values are mean $\pm$ SE mean ($n=5$).

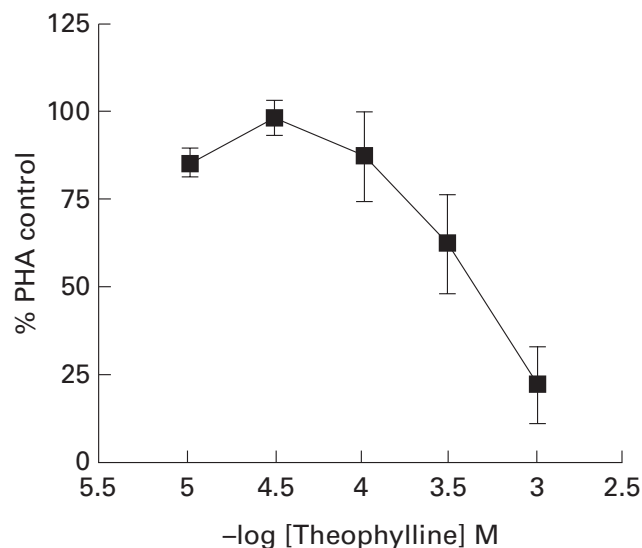


Fig. 9. Effect of theophylline on the PHA ($2\text{ }\mu\text{g/mL}$) stimulated proliferation of umbilical cord blood mononuclear cells. Data are expressed as percentage of the values obtained for cells stimulated with PHA in the absence of drug. Values are mean $\pm$ SE mean ($n=5$).

including macrophages, eosinophils and T-lymphocytes [25]. The type 4 PDE isoenzyme appears to be the main PDE isoenzyme present in inflammatory cells and has been characterized in T-lymphocytes, eosinophils, neutrophils, monocytes, basophils, mast cells and endothelial cells [15,26], although the type 3 and type 7 PDE isoenzymes have also been identified in human peripheral blood lymphocytes [4,16,18].

The present results show that the type 1 inhibitor, vinpocetine, the type 3 inhibitor, siguazodan, and the PDE5 inhibitor, zaprinast, had no effect on the PHA-stimulated proliferation of cord blood cells, suggesting that the type 1, 3 and 5 isoenzymes are not involved in the control of proliferation.

The type 4 PDE inhibitors, rolipram, RO 20-1724 and CDP840, however, produced a concentration-related inhibition of PHA-stimulated mononuclear cell proliferation. This is similar to the way in which adult mononuclear cells respond to PDE4 inhibitors [1,27].

The type 4 PDE inhibitors, rolipram, RO 20-1724 and CDP840, produced between 50 and 80% inhibition of the proliferative response of cord blood mononuclear cells at $10\text{ }\mu\text{mol/L}$ but were unable to inhibit proliferation completely. The mixed type 3/4 PDE inhibitor, zardaverine, suppressed proliferation by about 85% at a concentration of $10\text{ }\mu\text{mol/L}$.

It is not clear at which stage in the proliferation process the PDE inhibitors are acting to inhibit proliferation. T-cell

activation and proliferation requires two initial signals. PHA stimulates T-cells to pass from the G₀ phase to the G₁ phase of the cell cycle, and to synthesize protein [28]. A second signal, interleukin 1 (IL-1), which is produced by adherent accessory cells, is required to initiate DNA synthesis and acts on primed T-lymphocytes to induce the production of interleukin 2 (IL-2) and the appearance of IL-2 receptors [29]. Activated T-lymphocytes then proliferate in response to IL-2 [30]. The PDE inhibitors could therefore either be acting at the level of the accessory cells to block IL-1 release, or they could be having a direct effect on the lymphocytes to inhibit proliferation.

If the drugs are inhibiting proliferation by a direct effect on lymphocytes, then there are a number of stages of the proliferation process at which they could be acting. They could be having an inhibitory effect on IL-2 production. Some studies have demonstrated that PDE4 inhibitors can affect cytokine production. For example, PDE 4 inhibitors have been shown to inhibit TNF, IL-1 and IL-2 generation from monocytes and lymphocytes [2,12].

There is also evidence to suggest from several studies that patients on long-term theophylline therapy have an increase in the number and activity of T suppressor cells [31–33]. These theophylline-sensitive cells have been shown to suppress peripheral blood mononuclear cell proliferation [34]. It would therefore also seem plausible that the type 4 PDE inhibitors could be having a stimulatory effect on T suppressor cells. This is of interest, given that theophylline has recently been suggested to possess immunomodulatory activity in asthmatic subjects [35,36].

Lastly, it is possible that the drugs are having a cytotoxic effect, as there is evidence to suggest that high levels of cAMP are cytotoxic. However, as the inhibition by PDE isoenzyme inhibitors did not reduce [³H]-thymidine incorporation to below that in unstimulated cultures, this would suggest that cell viability was not being affected.

In conclusion, these data suggest that PDE4 is present in umbilical cord mononuclear cells and that inhibition of PDE4 can suppress PHA-stimulated umbilical cord blood mononuclear cell proliferation.

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