

Allergen challenge alters lymphocyte phosphodiesterase activity in horses with heaves

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Received 23 September 2003; revised 30 January 2004; accepted 16 February 2004

Abstract

Heaves is an allergic airway disease in horses characterised by reversible airway obstruction, bronchial hyperresponsiveness and airway inflammation associated with a Th₂ response. Cyclic nucleotide-dependent signalling pathways can regulate lymphocyte function. In this study, we examined lymphocyte PDE activity comparing horses with heaves to healthy control animals. Total PDE activity and the effects of isoenzyme selective inhibitors were measured before, 5 and 24 h after the start of a 7 h allergen challenge. Allergen challenge had no effect on either total cAMP PDE activity or its inhibition by the PDE4 selective inhibitor, rolipram, and the non-selective PDE inhibitor, theophylline. In contrast, the PDE3 selective inhibitor, quazinson, caused significantly greater inhibition of cAMP PDE activity before challenge in the heaves susceptible group. Additionally, total cGMP PDE activity was significantly lower 24 h after the start of challenge in the heaves affected group (11 ± 2 and 21 ± 3 pmol/min/mg for heaves and control animals, respectively) and the PDE5 selective inhibitor, zaprinast, caused significantly less inhibition in the heaves group at this time point. The functional significance of these findings was explored by examining the effect of PDE3, PDE4 and PDE5 selective inhibitors on mitogen-induced mononuclear cell proliferation before and 24 h after the start of allergen challenge. Proliferation decreased after challenge in the heaves group (stimulation index = 328 ± 110 and 200 ± 72 before and after challenge, respectively) whilst remaining constant in the control group (stimulation index = 161 ± 13 and 183 ± 45 before and after challenge, respectively). However, all three PDE inhibitors caused a similar amount of inhibition at each time point and the effect of a combination of a PDE3 and a PDE5 inhibitor was simply additive in both groups. These results suggest differences in the control of lymphocyte PDE activity in horses with heaves.

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Keywords: Phosphodiesterase; Lymphocyte; Heaves; Asthma; Proliferation

1. Introduction

Heaves is an allergic respiratory condition characterised by acute, reversible bronchoconstriction, bronchial hyperresponsiveness and neutrophil recruitment to the lungs [1]. Affected horses develop signs 4–5 h after the start of exposure to poor quality forage and bedding [2] suggesting that the response is similar to the late asthmatic response in man with the allergens involved being fungal spores and actinomycetes. In addition to neutrophil recruitment to

the airways, perivascular and peribronchiolar accumulation of lymphocytes occurs [3] and results from a study by McGorum et al. [4] suggest that, on exposure to allergen, susceptible horses show an increase in the proportion of CD4⁺ cells in bronchoalveolar lavage fluid. More recently it has been shown that, as with the asthma phenotype, horses with heaves have a predominant Th₂-type cytokine profile [5].

Cyclic AMP-dependent signalling pathways have been shown to be regulatory in inflammatory cells in man and other species [6–9]. Cyclic nucleotides are hydrolysed by phosphodiesterases (PDEs), a growing family of isoenzymes of which 11 have been identified and classified according to their localisation, substrate specificity and sensitivity to endogenous and exogenous substances [10–12]. Using isoenzyme selective inhibitors, equine lymphocytes have

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been shown to contain PDE1, PDE3, PDE4 and PDE5 [13], a similar PDE profile to that of human lymphocytes [8,14], although a PDE7 selective inhibitor was not available to determine whether this isoenzyme is present in equine cells. In addition, inhibitors selective for PDE3, PDE4 and PDE5 attenuate mitogen-induced equine peripheral blood mononuclear cell proliferation (PBMC) [15].

Biochemical and functional differences in PDE have been described in lymphocytes and monocytes from human subjects with asthma or atopic dermatitis [16–20]. The observed differences include increased PDE activity in mononuclear cells from asthmatic subjects and atopic dermatitis patients [16,17,20] and altered sensitivity to inhibition by PDE isoenzyme selective inhibitors in mononuclear cells from asthmatic subjects and atopic dermatitis patients [16,17]. PDE isoenzymes are known to be involved in the regulation of lymphocyte proliferation and cytokine production [8,9] and cytokines from activated T cells are likely to play a part in the neutrophil recruitment and activation in the airways of horses with heaves and certain populations of asthmatic subjects [1,5,21–23]. The first aim of this study was to determine if there were differences in PDE activity or its sensitivity to inhibition by isoenzyme selective inhibitors in lymphocytes from horses with heaves both in remission and following antigen-induced disease when compared to normal animals. The functional relevance of the observed differences was then examined by comparing the effects of PDE inhibitors on mitogen-induced mononuclear cell proliferation in healthy and allergic horses.

2. Methods

2.1. Animals

The lymphocyte PDE isoenzyme profile of six animals selected from a group of eight mixed breed heaves-susceptible horses and ponies in remission (age range 8–24 yr) was first determined to establish whether cells from allergic horses had a similar profile to that of healthy controls. Subsequently five animals from the heaves group (age range 8–18 yr) and five age-matched healthy New Forest ponies were used in the allergen challenge and mononuclear cell proliferation studies. The heaves group consisted of New Forest and Welsh Mountain ponies and Warmblood horses. Prior to antigen challenge all the animals used in the study were kept in an allergen-free environment and received no medication except for routine vaccination and worming. Horses in the heaves affected group had previously been diagnosed by demonstrating a pleural pressure within normal limits (≤ 5 cm H₂O) and $< 10\%$ BAL neutrophils when in remission. Following a 7 h exposure to allergen, an increase in pleural pressure corresponding to ≥ 2 cm H₂O with an absolute value > 5 cm H₂O and a BAL differential cell count of $\geq 40\%$ neutrophils at 24 h occurred. Control animals showed no

difference in pleural pressure and BAL differential cell count following allergen challenge. For the purpose of this study, pleural pressure was deemed to be sufficient indication of disease. This work was carried out under a Home Office project licence that had been approved by the Royal Veterinary College Ethics and Welfare Committee.

2.2. Allergen challenge

Animals were stabled for 7 h in a closed airspace containing 20 kg poor quality hay, which was shaken hourly to aid fungal spore dispersal. Pleural pressure was measured prior to and 7 and 24 h after the start of challenge using an oesophageal balloon catheter as previously described [2]. The level of dust exposure was recorded using a Vortex sampling pump set to a flow rate of 2.0 l/min connected to an Institute of Occupational Medicine sampling head capable of measuring total inhaled dust (Casella, Bedford, UK). Prior to challenge, a pre-weighed glass fibre filter was inserted into the sampling head, which was attached to the horse's head approximately 2 in. away from the nostril. The filter was re-weighed at the end of the challenge period and dust exposure calculated as mg/h. After the 7 h challenge, animals were returned to an allergen-free environment.

2.3. Lymphocyte purification

Lymphocytes were purified from venous blood (150–200 ml) collected into 0.4 M ethylenediaminetetraacetic acid (EDTA) as previously described [15]. Briefly, PBMCs were separated by Percoll density centrifugation, washed in Hank's balanced salt solution and re-suspended at 5×10^6 cells/ml in RPMI 1640 containing 2 mM L-glutamine and 10% heat inactivated foetal calf serum. Cells were cultured for 1 h at 37 °C in 5% CO₂, after which non-adherent cells were removed and counted. Cells were subsequently lysed in homogenisation buffer (leupeptin 10 µg/ml, Tris (pH 8.0) 20 mM, MgCl₂ 2 mM, dithiothreitol 1 mM, EDTA 5 mM, benzamidine 1.3 mM, sucrose 0.25 M, N α -p-tosyl-L-lysine chloromethyl ketone 20 µM, triton X-100 1%; v/v) to give approximately 3×10^7 cells/ml, vortexed and stored at -80 °C prior to analysis of PDE activity. In order to confirm the purity of the cell population, 8×10^6 cells were re-suspended in phosphate buffered saline containing 0.2% bovine serum albumin and examined by flow cytometry using a panel of monoclonal antibodies that recognise equine cells expressing CD3, CD4, CD8, CD13, MHC class I, MHC class II and an immunoglobulin antibody, B29A, which specifically binds to equine B lymphocytes [24] as previously described [15].

2.4. Measurement of PDE activity

PDE activity was measured using a two-step radioactivity assay as previously described for equine

neutrophils [25] and expressed as pmol hydrolysed cyclic nucleotide/min/mg protein. The protein content of the samples was measured using the colorimetric method described by Lowry et al. [26] For the initial isoenzyme analysis study, aliquots containing approximately 1×10^6 homogenised cells were assayed for PDE activity in the presence of isoenzyme selective inhibitors for PDE1, PDE3, PDE4 and PDE5 and cGMP, which stimulates PDE2. All compounds were added at a single concentration at least 10-fold higher than the documented IC_{50} for inhibition of the purified isoenzyme in other species whilst still conferring selectivity [27,28]. For the allergen challenge study, lymphocytes were purified and lysed from five heaves susceptible and five control horses prior to and 5 and 24 h after the start of challenge. These time points were chosen because lung function changes and neutrophil recruitment to the lungs have been shown to occur after 5 h of allergen exposure and activation of circulating neutrophils has been demonstrated at 24 h [2,29]. Thus, if lymphocyte activation is involved in either recruitment or activation of circulating neutrophils then changes in cyclic nucleotide signalling may be evident at these time points. In order to explore this possibility, total cAMP and cGMP PDE activity was measured and the effects of increasing concentrations of the non-selective inhibitor, theophylline (1×10^{-7} to 1×10^{-3} M), the PDE4 inhibitor, rolipram (1×10^{-9} to 1×10^{-4} M), the PDE3 inhibitor, quazinsonone (1×10^{-9} to 1×10^{-4} M) and the PDE5 inhibitor, zaprinast (1×10^{-9} to 1×10^{-4} M), were examined. In view of the high concentration of vinpocetine required to produce a relatively small inhibitory effect and its poor solubility, the PDE1 inhibitor was not included in this study. To minimise inter-assay variability samples taken from one animal in each group were analysed together.

2.5. PBMC proliferation

PBMC proliferation was measured using cells from five heaves susceptible and five control horses. PBMCs were separated by Percoll density centrifugation before and 24 h after the start of allergen challenge from 50 ml venous blood collected into heparin (20 iu/ml) and re-suspended at 1×10^6 cells/ml in RPMI 1640 containing 10% autologous horse plasma, 2 mM L-glutamine, 50 μ g/ml streptomycin and 50 iu/ml penicillin. Cells (5×10^5) were incubated for 72 h at 37 °C in 5% CO₂ with a concentration of phytohaemagglutinin (PHA) that caused 90% maximal proliferation (56 μ g/ml) in the presence of rolipram (1×10^{-8} to 1×10^{-4} M), the PDE3 inhibitor, milrinone (1×10^{-7} to 1×10^{-4} M) or zaprinast (1×10^{-7} to 1×10^{-4} M). In addition, the effect of a combination of milrinone (1×10^{-5} M) and zaprinast (1×10^{-5} M) was also examined. Milrinone was used in preference to quazinsonone in this study as the compound demonstrated better solubility. Both compounds inhibit equine PBMC proliferation to a similar degree [15]. Proliferation was

measured by [³H]-thymidine incorporation as previously described [30]. All compounds were dissolved in DMSO and serial dilutions were made in RPMI 1640 containing 2 mM L-glutamine, 50 μ g/ml streptomycin and 50 iu/ml penicillin to ensure that the final concentration of DMSO in contact with the cells did not exceed 0.1% except for the combination of milrinone and zaprinast where the final concentration of DMSO was 0.2%.

2.6. Materials

Sodium heparin was obtained from National Veterinary Supplies, Staffordshire, UK. [³H]-cAMP and [³H]-cGMP were obtained from Amersham Pharmacia Biotech, Buckinghamshire, UK. [³H]-thymidine was obtained from ICN Pharmaceuticals Ltd, Hampshire, UK. PHA was obtained from Biostat, Derbyshire, UK. RPMI 1640 and penicillin–streptomycin (5000 iu/ml–5000 μ g/ml) were obtained from Invitrogen, Renfrewshire, UK. Methanol was obtained from Merck Ltd, Leicestershire, UK. Rolipram was kindly provided by Schering AG, Berlin, Germany. Zaprinast, quazinsonone, milrinone and vinpocetine were obtained from Calbiochem, Nottinghamshire, UK. Foetal calf serum was obtained from Harlan Sera-Lab, Leicestershire, UK. FACS isotype negative controls and antibodies recognising CD13, MHC class I and II were obtained from Serotec Ltd, Oxfordshire, UK. The antibody recognising B cells were obtained from VMRD, Inc., Washington, USA. The antibody recognising CD3 was kindly provided by Prof J. Stott, University of California, California, USA. The antibody recognising CD4 was kindly provided by Dr M. Holmes, Cambridge, UK. The antibody recognising CD8 was kindly provided by S. Mayall, Royal Veterinary College, London, UK. The FITC-conjugated rabbit anti-mouse immunoglobulin F(ab)₂ was obtained from DAKO Ltd, Cambridgeshire, UK. All other reagents were obtained from Sigma Diagnostics, Dorset, UK.

2.7. Data analysis

All data are presented as mean $\pm$ sem. To calculate lymphocyte purity following separation, the total number of CD3 and B29A positive cells was expressed as a percentage of the number of cells staining positive for MHC class I as this molecule is expressed on all equine leucocytes [24]. PBMC proliferation was expressed as a stimulation index by calculating mean disintegrations per minute (dpm) in the presence of PHA and dividing by mean dpm in the presence of medium alone. For the allergen challenge studies, dust exposure and increases in pleural pressure were compared between heaves susceptible and control animals using Student's unpaired *t*-test (GraphPad Prism3, GraphPad Software, San Diego, USA). The effects of single high concentrations of PDE isoenzyme inhibitors were analysed by repeated measures one-way ANOVA (GraphPad Prism3). Any significant differences were investigated

further by comparing PDE activity in the presence of isoenzyme selective compounds to total PDE activity using Dunnett's post hoc test. The effects of allergen challenge on lymphocyte total cAMP and cGMP PDE activity and PHA-induced PBMC proliferation were compared in cells isolated from heaves susceptible and control animals using repeated measures two-way ANOVA (GraphPad Prism3). Any significant interactions were noted and significant differences were investigated further using Bonferroni's post hoc test. The concentration-dependent effects of the PDE inhibitors on both PDE activity and PBMC proliferation were compared within each group at the different time points using ANCOVA with concentration as the covariate (Minitab, Minitab, Inc., PA, USA). Any significant differences were investigated further by comparing the effect of the inhibitor between the two groups at each time point (ANCOVA). Differences were considered significant when a *p* value of 5% or less was found.

3. Results

3.1. Allergen challenge

All the horses with heaves responded with a significant increase in pleural pressure following a 7 h exposure to poor quality hay. In contrast, no such increase occurred in the control group (Table 1a). There was no significant difference in the amount of dust that each group of animals was exposed to in either of the allergen challenge studies (Table 1b).

3.2. Lymphocyte populations

FACS analysis demonstrated that there was no difference in the purity of the cell populations obtained from the two groups of animals with 84 ± 2 and $77 \pm 1\%$ T cells and 7 ± 1 and $9 \pm 1\%$ B cells recovered from heaves and control animals, respectively. The total number of cells staining positive for lymphocyte markers was 91 ± 2 and $86 \pm 2\%$ for heaves and control animals, respectively ($p = 0.1$; Student's unpaired *t*-test). Although a platelet marker was not included in the FACS analysis, examination of a modified Wright's-stained smear under high power showed no visible evidence of platelets in cells purified from either heaves or control animals.

Table 1a
Increase in pleural pressure following a 7 h allergen challenge

	Control (cm H ₂ O)	Heaves (cm H ₂ O)	<i>p</i> value
Challenge 1	-0.3 ± 0.7	2.8 ± 0.3	0.003
Challenge 2	0.2 ± 0.3	3.5 ± 0.8	0.005

Results are expressed as mean increase at 7 h $\pm$ sem. Data are from two separate challenges with five animals in each group. Values for the control and heaves groups were compared by Student's unpaired *t*-test; $p < 0.05$ was considered significant.

Table 1b
Dust exposure during a 7 h allergen challenge

	Control (mg/h)	Heaves (mg/h)	<i>p</i> value
Challenge 1	0.8 ± 0.1	1.2 ± 0.2	0.09
Challenge 2	1.1 ± 0.1	1.0 ± 0.1	0.5

Results are expressed as mean $\pm$ sem. Data are from two separate challenges with five animals in each group. Values for the control and heaves groups were compared by Student's unpaired *t*-test; $p < 0.05$ was considered significant.

3.3. PDE isoenzyme analysis

The isoenzyme profile of lymphocytes isolated from six heaves susceptible horses is shown in Fig. 1. All the isoenzyme selective compounds tested inhibited cAMP PDE activity demonstrating the presence of PDE1, PDE3 and PDE4. Addition of cGMP also caused inhibition producing further support for the presence of PDE3 as cGMP stimulates PDE2 activity but inhibits PDE3. However, as the PDE3 inhibitor, quazinson, caused more inhibition of cAMP hydrolysis than cGMP, the presence of PDE2 cannot be ruled out. Addition of the PDE5 inhibitor, zaprinast, significantly inhibited cGMP PDE activity, indicating the presence of this isoenzyme in lymphocytes from heaves susceptible horses.

3.4. Effect of allergen challenge on PDE activity

Total cAMP PDE activity was not affected by allergen challenge with a similar amount of activity being found in cells from both groups of animals at all the time points

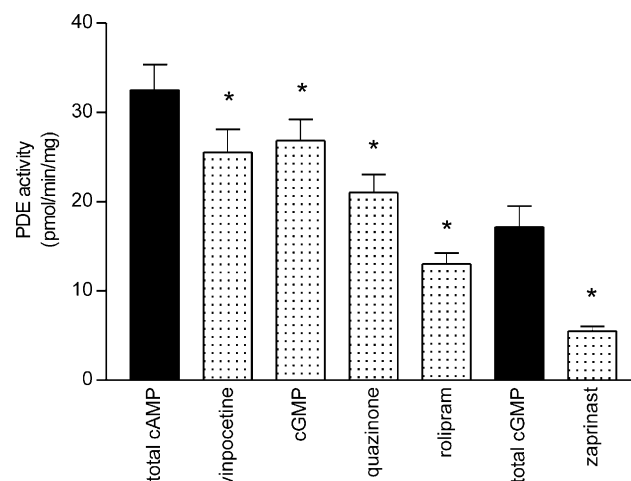


Fig. 1. cAMP and cGMP PDE isoenzyme profile of lymphocytes isolated from heaves susceptible horses. Filled columns represent total PDE activity. Vinpocetine (1×10^{-4} M) inhibits PDE1, cGMP (5×10^{-6} M) stimulates PDE2 and inhibits PDE3, quazinson (6×10^{-5} M) inhibits PDE3, rolipram (1×10^{-5} M) inhibits PDE4 and zaprinast (1×10^{-5} M) inhibits PDE5. Columns represent mean values and bars represent sem ($n = 6$). Data were analysed by repeated measures one-way ANOVA followed by Dunnett's post hoc test. Significant differences between responses in the presence of inhibitors and the relevant control are indicated by an asterisk (* $P < 0.05$).

examined (Fig. 2a). In contrast, total cGMP PDE activity was influenced by challenge with significantly less activity measured in lymphocytes from heaves affected horses 24 h after the start of allergen challenge when compared to that found in cells from control animals (Fig. 2b).

All the inhibitors tested produced concentration related inhibition of PDE activity in both heaves and control animals. Allergen challenge did not alter the inhibitory effects of the non-selective inhibitor, theophylline (Fig. 3), or the PDE4 selective inhibitor, rolipram (Fig. 4), in lymphocytes from either group of horses. In contrast, the inhibitory effect of the PDE3 selective inhibitor, quazone, was significantly greater before challenge than at 5 and 24 h after challenge in the heaves group (Fig. 5a) whereas a similar amount of inhibition was seen at all time points in the control group (Fig. 5b). Moreover, before challenge significantly greater inhibition occurred in the heaves group when compared to that in the control group (Fig. 5c) whereas similar inhibitory effects were observed

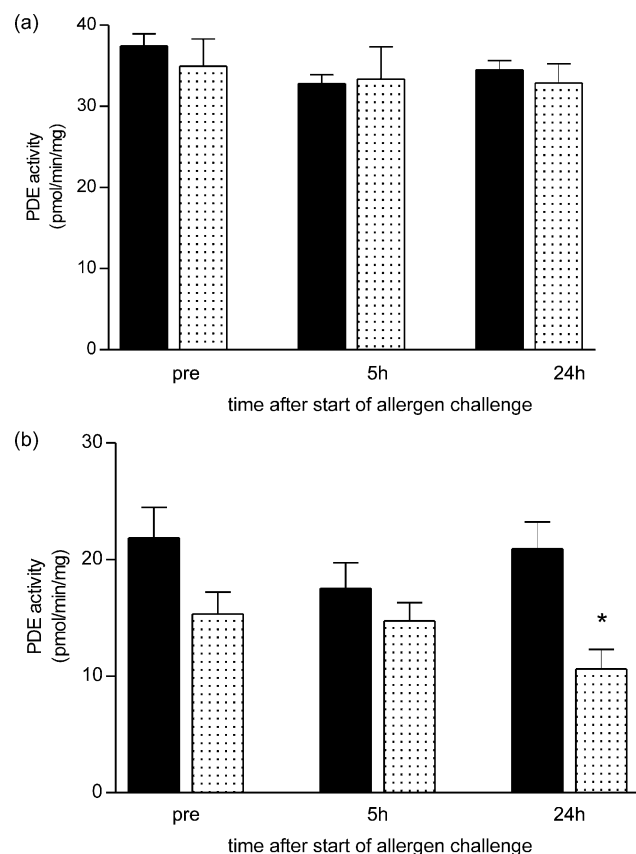


Fig. 2. Effect of allergen challenge on (a) total cAMP PDE activity and (b) total cGMP PDE activity in equine lymphocytes isolated from control (filled columns) and heaves affected (dotted columns) animals before and 5 and 24 h after the start of challenge. Columns represent mean values and bars represent sem ($n = 5$). Data were analysed by repeated measures two-way ANOVA followed by Bonferroni's post hoc test. Significant differences between control and heaves affected animals at each time point are indicated by an asterisk (* $P < 0.05$).

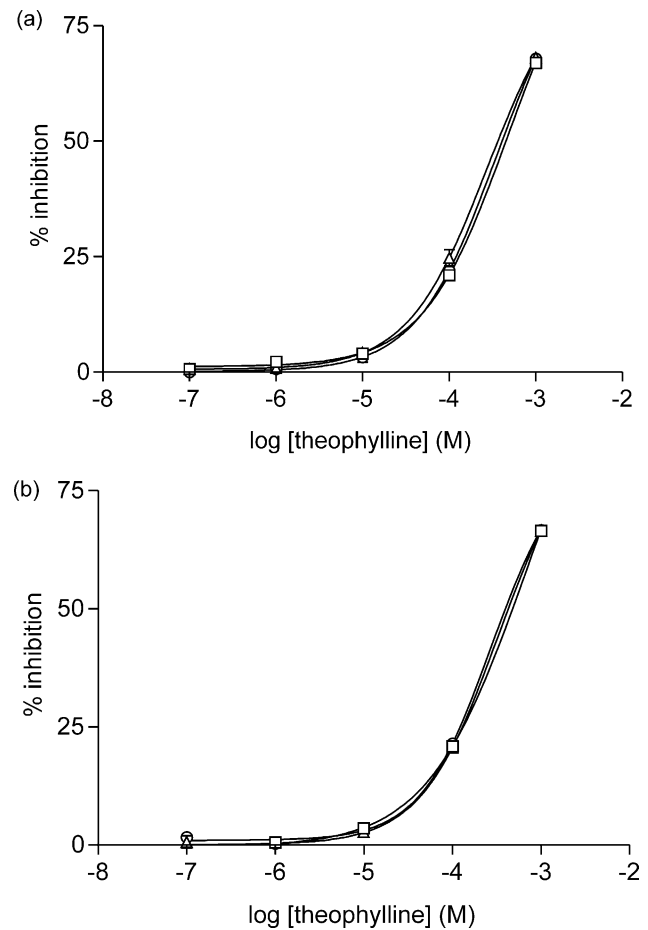


Fig. 3. cAMP PDE activity was measured in cells from (a) control animals and (b) heaves affected animals in the presence of the non-selective PDE inhibitor, theophylline (1×10^{-7} to 1×10^{-3} M) before (square symbol), 5 h (triangle symbol) and 24 h (circle symbol) after the start of a 7 h allergen challenge. Results are expressed as %inhibition of PDE activity in the presence of drug vehicle (0.1% DMSO). Points represent mean values and bars represent sem ($n = 5$). Data were analysed by ANCOVA and no significant differences in the response to theophylline were found.

between the groups at both 5 and 24 h after challenge (data not shown).

When comparing the inhibitory effects of the PDE5 selective inhibitor, zaprinast, significantly less inhibition occurred in lymphocytes from the heaves group 24 h after the start of challenge (Fig. 6a) whereas a similar degree of inhibition occurred at all time points in the control group (Fig. 6b). When the effect of zaprinast was compared between the groups at each time point, there was significantly less inhibition in lymphocytes from the heaves group only at 24 h (Fig. 6c). Similar inhibitory effects were observed between the groups both before and at 5 h after the start of challenge (data not shown).

3.5. Effect of allergen challenge on PBMC proliferation

The effect of allergen challenge on PHA-induced PBMC proliferation was significantly different in the two groups

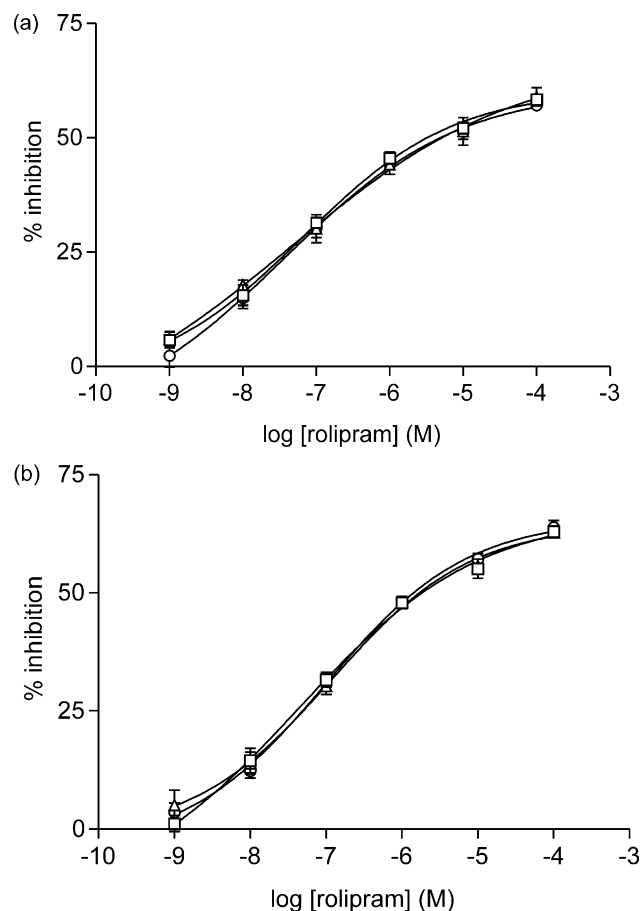


Fig. 4. cAMP PDE activity was measured in cells from (a) control animals and (b) heaves affected animals in the presence of the PDE4 inhibitor, rolipram (1×10^{-9} to 1×10^{-4} M) before (square symbol), 5 h (triangle symbol) and 24 h (circle symbol) after the start of a 7 h allergen challenge. Results are expressed as %inhibition of PDE activity in the presence of drug vehicle (0.1% DMSO). Points represent mean values and bars represent sem ($n = 5$). Data were analysed by ANCOVA and no significant differences in the response to rolipram were found.

as indicated by a p value of 0.02 for the interaction between time and group in the repeated measures two-way ANOVA carried out to analyse the data. Fig. 7 shows that the proliferation index was similar before and 24 h after the start of challenge in the control group whilst PBMCs from heaves susceptible horses behaved differently over time showing a decrease in proliferation after challenge. However, ANCOVA showed that over the concentration range tested, there was no significant difference in the ability of PDE3, PDE4 and PDE5 selective inhibitors to attenuate proliferation in either group 24 h after challenge when compared with pre-challenge values. Table 2 shows that the percentage inhibition of PDE activity when each inhibitor was added at 1×10^{-5} M was similar for both groups before and 24 h after challenge. Additionally incubation of PBMCs with a combination of a PDE3 (1×10^{-5} M) and a PDE5 (1×10^{-5} M) selective inhibitor resulted in a similar degree of inhibition of proliferation in both groups before and 24 h after the start of challenge (Table 2).

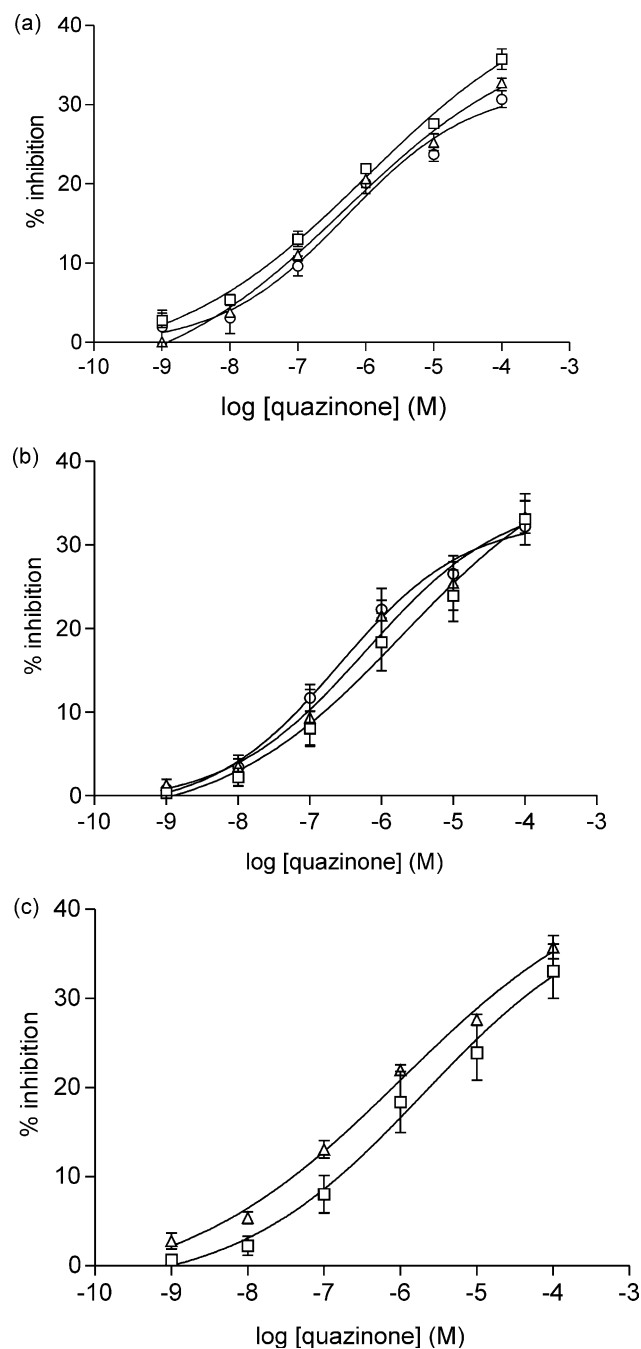


Fig. 5. cAMP PDE activity was measured in cells from (a) heaves affected animals and (b) control animals in the presence of the PDE3 inhibitor, quazinsonone (1×10^{-9} to 1×10^{-4} M) before (square symbol), 5 h (triangle symbol) and 24 h (circle symbol) after the start of a 7 h allergen challenge. (c) The effect of quazinsonone on PDE activity in cells from control (square symbol) and heaves affected (triangle symbol) animals before challenge. Results are expressed as %inhibition of PDE activity in the presence of drug vehicle (0.1% DMSO). Points represent mean values and bars represent sem ($n = 5$). The inhibitory effect of quazinsonone was significantly greater before challenge in cells from the heaves affected animals when compared to 5 and 24 h after challenge (ANCOVA; $p < 0.05$). Significantly greater inhibition was found in lymphocytes from the heaves group before challenge when compared to cells from control animals (ANCOVA; $p < 0.05$).

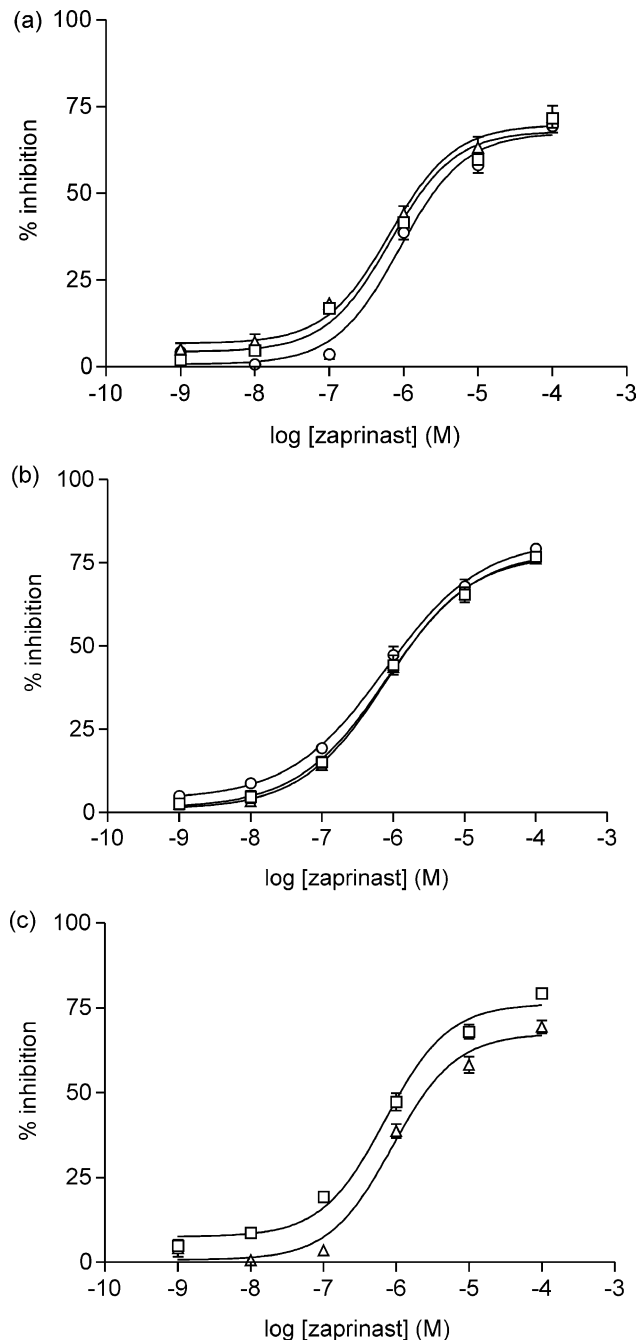


Fig. 6. cGMP PDE activity was measured in cells from (a) heaves affected animals and (b) control animals in the presence of the PDE5 inhibitor, zaprinast (1×10^{-9} to 1×10^{-4} M) before (square symbol), 5 h (triangle symbol) and 24 h (circle symbol) after the start of a 7 h allergen challenge. (c) The effect of zaprinast on PDE activity in cells from control (square symbol) and heaves affected (triangle symbol) animals 24 h after challenge. Results are expressed as %inhibition of PDE activity in the presence of drug vehicle (0.1% DMSO). Points represent mean values and bars represent sem ($n = 5$). The inhibitory effect of zaprinast was significantly lower at 24 h after challenge in cells from the heaves affected animals when compared to before and 5 h after challenge (ANCOVA; $p < 0.05$). Significantly less inhibition was found in lymphocytes from the heaves group 24 h after challenge when compared with cells from control animals (ANCOVA; $p < 0.05$).

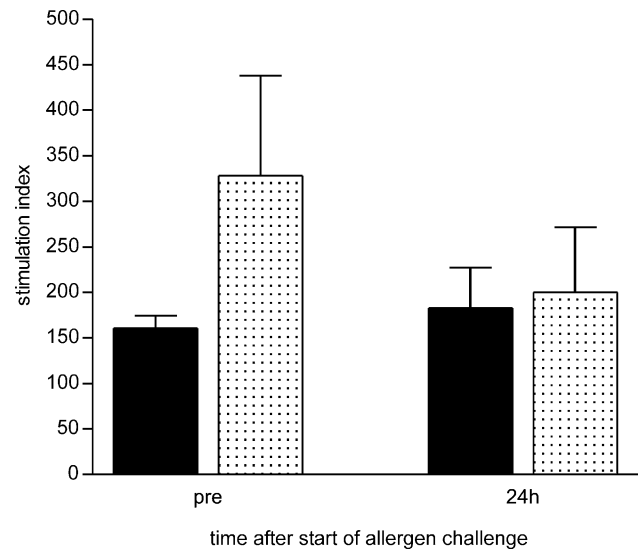


Fig. 7. PHA-induced proliferation of PBMCs isolated from control (filled columns) and heaves affected (dotted columns) animals before and 24 h after the start of allergen challenge. Results are expressed as a stimulation index (proliferation in the presence of PHA/proliferation in the presence of vehicle). Mean disintegrations per minute for vehicle treated cells = 606 ± 85 and 658 ± 132 for control animals and 394 ± 176 and 700 ± 313 for heaves-susceptible animals before and 24 h after challenge, respectively. Columns represent mean values and bars represent sem ($n = 5$). A significant interaction between time and group was found (repeated measures two-way ANOVA; $p = 0.02$).

4. Discussion

Alterations in cyclic nucleotide signalling pathways have been shown to occur in allergic disease as a result of a primary biochemical abnormality [31] or as a direct consequence of the inflammatory response induced by allergen exposure [32]. In this study, we compared PDE activity and its inhibition in lymphocytes from asymptomatic heaves susceptible horses and healthy, age matched controls and examined the effect of allergen exposure. It was

Table 2

Effect of allergen challenge on PHA-induced proliferation of PBMCs isolated from control and heaves affected horses in the presence of isoenzyme selective inhibitors of PDE3 (milrinone), PDE4 (rolipram) and PDE5 (zaprinast)

	Group	%Inhibition	
		Pre	24 h
Milrinone	Control	31 ± 3	26 ± 4
	Heaves	27 ± 1	25 ± 3
Rolipram	Control	68 ± 1	66 ± 2
	Heaves	62 ± 3	64 ± 4
Zaprinast	Control	33 ± 2	31 ± 2
	Heaves	34 ± 6	30 ± 4
Milrinone/zaprinast	Control	45 ± 3	51 ± 4
	Heaves	45 ± 3	47 ± 2

Results are expressed as mean $\pm$ sem %inhibition achieved with each of the inhibitors at 1×10^{-5} M before and 24 h after the start of challenge ($n = 5$).

not possible to use identical breeds and so breed-related differences cannot be ruled out. However, a previous study of equine neutrophil PDE indicated that breed is unlikely to affect PDE activity.

Total cAMP and cGMP PDE activity were found to be similar in lymphocytes from heaves susceptible horses and healthy animals prior to allergen challenge. This is in contrast to the findings of a study by Tohda et al. [20] who reported that total PDE activity in lymphocytes from human asthmatic subjects was higher than that in cells from normal subjects. The difference between the data obtained in allergic equine and human subjects may reflect species variability. Alternatively, PDE activity in human cells may have been influenced by prior exposure to beta agonists or theophylline [20,33,34], whereas none of the heaves susceptible horses had received any medication. Lymphocytes from heaves susceptible horses were also shown to contain the same PDE isoenzymes as those in cells from normal animals [13]. It should be noted, however, that the presence of PDE1 was investigated in the absence of calcium and calmodulin. Although PDE1 is known to be activated by these substances, there is evidence to show it is still active in their absence [35]. In this study, the small effect of vinpocetine is, therefore, likely to be due to inhibition of residual PDE1 activity.

Allergen challenge of heaves susceptible horses had no effect on lymphocyte total cAMP PDE activity, which is in agreement with data from Landells et al. [36] who found no difference in total cAMP PDE activity in monocytes from human asthmatic subjects before and at 24 h after allergen challenge. In view of the higher levels of cGMP PDE activity found in equine, when compared with human, lymphocytes [13,37], total cGMP PDE activity was also measured before and after allergen challenge. Interestingly, lymphocytes from horses with heaves had a significantly lower amount of activity 24 h after challenge when compared with cells isolated from the control group. In addition, following allergen challenge, the PDE5 inhibitor, zaprinast, caused significantly less inhibition of cGMP PDE activity in the heaves group when compared with the control group at 24 h. This was confirmed as a real decrease over time by comparing the effects of the inhibitor within each group of horses at the three time points studied. Therefore, the decrease in total cGMP PDE activity 24 h after allergen challenge in horses with heaves could be attributed to a reduction in PDE5 activity. Changes in platelet and B cell numbers could have influenced the effects of zaprinast [38,39]. However, platelets were not detected in the samples and, although B cell numbers were not determined, others have shown no difference in blood lymphocyte phenotype ratios of control and heaves affected horses either before or after antigen challenge [4]. In view of the difference between the size of the reduction in total cGMP PDE activity and that of PDE5 activity, it is possible that the activity of other cGMP hydrolysing enzymes is also decreased at 24 h although the potency of zaprinast

as an inhibitor of PDE5 in the horse may contribute to the observed discrepancy.

The inhibitory effects of quazinone were significantly greater in lymphocytes isolated from the heaves susceptible horses before challenge when compared with the control group. This was confirmed as a real increase by comparing the amount of inhibition at the three time points within each group of horses and might indicate an inherent defect in PDE3 regulation in lymphocytes from horses susceptible to allergic airway disease. PDE3 is regulated by cGMP, which inhibits enzyme activity [10]. However, there was no difference in total cGMP PDE activity in lymphocytes from the two groups of horses prior to challenge, which suggests that other PDE3 regulatory mechanisms are altered. In contrast, the decrease in cGMP PDE activity observed 24 h after challenge in lymphocytes isolated from heaves affected horses could explain why the PDE3 inhibitory activity of quazinone was lower at 24 h in the heaves affected group relative to pre-challenge values.

In view of the fact that total cAMP PDE activity was similar in both groups, and was not affected by allergen challenge, the increased inhibition of PDE3 activity is likely to be related to a reciprocal decrease in the activity of another cAMP hydrolysing isoenzyme. Landells et al. [16] found that the PDE3 inhibitor, siguazodan, caused significantly greater inhibition of monocyte PDE activity in cells isolated from asthmatic subjects whilst the PDE4 inhibitor, rolipram, caused significantly less inhibition. However, in equine lymphocytes rolipram caused a similar amount of inhibition in both groups prior to challenge suggesting that in equine cells another cAMP hydrolysing isoenzyme may be down regulated. Human lymphocytes are known to contain PDE7 [37]. However, until very recently, no PDE7 selective inhibitors have been available for use [40] and the role of this isoenzyme has yet to be explored in equine cells. As the PDE4 inhibitor, rolipram, caused a similar amount of inhibition in cells from control and heaves susceptible horses, and allergen challenge did not affect the amount of inhibition observed, PDE4 activity in equine lymphocytes does not appear to be affected by either susceptibility to allergic airway disease or clinical disease. This is in agreement with the results of Landells et al. [36] who found no difference in PDE4 subtype mRNA expression in lymphocytes isolated from healthy and asymptomatic asthmatic subjects.

Antigen challenge had a differential effect on mitogen-induced PBMC, the magnitude of the response decreasing after challenge in the heaves group whilst remaining at a similar level in the control group. This could be linked with the observed changes in PDE3 and PDE5 activity over time with a decrease in cGMP PDE activity at 24 h after allergen challenge resulting in an increase in cGMP-mediated inhibition of PDE3 and a corresponding attenuation of the proliferative response. To test this hypothesis, the effects of PDE3 and PDE5 selective inhibitors were investigated alone and in combination. However, only additive effects

were obtained and these were of a similar magnitude in the two groups of horses both before and after allergen challenge. This suggests, therefore, that cAMP and cGMP are independently involved in modulating the proliferative response rather than elevated cGMP having a reciprocal inhibitory effect on cAMP PDE activity.

Milrinone, zaprinast and rolipram all caused a similar amount of inhibition of proliferation before and after challenge in both groups of horses. This suggests that, as has previously been shown in human monocytes [41], the biochemical difference identified in lymphocyte PDE isoenzyme activity in the heaves susceptible horses did not translate into an alteration in this particular cell function. Therefore, the decrease in total cGMP PDE activity at 24 h in the heaves group may explain the decrease in proliferative response whilst the lack of a significant difference of a PDE5 inhibitor at 24 h indicates that several different PDE isoenzymes are likely to be involved in the control of lymphocyte proliferation.

In conclusion, horses with allergic airway disease exhibit biochemical differences in lymphocyte PDE regulation, which may affect cell activation. These differences appear to be associated with both disease predisposition and disease status as they occur in horses in remission (increased PDE3 inhibitory activity) and following allergen challenge (decreased total cGMP PDE activity and PDE5 inhibitory activity). The observed differences are unlikely to be due to natural variation as no significant differences in total cAMP PDE activity or the inhibitory effects of a PDE4 inhibitor, or theophylline, a non-selective PDE inhibitor were observed before or after allergen challenge in either normal or heaves affected horses. In other allergic diseases, such as atopic dermatitis in man, the altered isoenzyme activity has been shown in some, but not all, studies to translate into increased sensitivity to inhibition of functional responses [8,17,19]. This has not been observed in horses with heaves using mitogen-induced proliferation as the indicator of cell responsiveness. As the effects of PDE inhibitors have been shown to be both stimulus and response specific [42], effects on other lymphocyte functions, such as cytokine production may be detected in future studies.

Acknowledgements

The authors would like to thank the Horserace Betting Levy Board for their financial support and the owners of the horses who kindly donated them for use in these studies.

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