# Evaluation of the effect of phosphodiesterase on equine platelet activation and the effect of antigen challenge on platelet phosphodiesterase activity in horses with recurrent airway obstruction

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**Objective**—To determine whether expression of equine platelet activation–dependent surface markers is influenced by phospodiesterase (PDE) isoenzyme activity and whether antigen challenge alters platelet PDE activity in horses with recurrent airway obstruction (RAO).

**Animals**—16 horses.

**Procedures**—7 healthy horses were used for in vitro experiments, 6 horses with RAO were used for antigen challenge, and 6 healthy horses were used as control animals. Three of the healthy horses had also been used in the in vitro experiments. Effects of PDE inhibition and activation of adenylyl cyclase on CD41/61 and CD62P expression on platelets and platelet-neutrophil aggregate formation in vitro were investigated via flow cytometry. Platelet PDE activity and sensitivity to inhibition of PDE3 and PDE5 isoenzymes were examined in horses with RAO and control horses before and after antigen challenge.

**Results**—Inhibition of PDE or activation of adenylyl cyclase significantly inhibited stimulus-induced expression of CD41/61 and CD62P (by approx 94% and 40%, respectively) and percentage of CD62P positive cells (by approx 30%). Only the PDE3 inhibitor, trequinsin, caused a significant (53%) reduction in platelet-neutrophil aggregate formation. Platelet PDE activity decreased following antigen challenge in RAO-affected horses and control horses. In horses with RAO, a significant increase in sensitivity of platelet PDE to inhibition by the PDE5 inhibitor zaprinast was observed after 5 hours.

**Conclusions and Clinical Relevance**—Results provided further evidence that PDE3 is an important regulator of equine platelet activation and suggested that changes in regulation of platelet PDE5 may contribute to antigen-induced response in horses with RAO. (*Am J Vet Res* 2010;71:534–540)

Platelet function is tightly regulated under physiologic conditions; however this tight control can become impaired in thrombotic and inflammatory disorders.[1] One mechanism by which the functional responses of these cells are controlled is through alterations in the intracellular concentrations of cAMP and cGMP.[2] This can be brought about by activation of adenylyl or guanylyl cyclase or by an alteration in the activity of PDE, which breaks down these cyclic nucleotides. Phosphodiesterases are a group of 11 families of metallophosphohydrolase enzymes differentiated by criteria including substrate specificity, genetic makeup, sensitivity to endogenous and exogenous regulators, and kinetics.[1–3] The distribution of PDE isoenzymes varies among cell types, and the isoenzymes in equine platelets, lymphocytes, and neutrophils have been characterized, although differentiation of the identified isoenzymes into further subgroups (isoforms) has not been attempted yet.[3–5]

Platelet activation is associated with increased expression of cell surface markers such as CD41/61 and

**Abbreviations**

| | |
|---|---|
| BALF | Bronchoalveolar lavage fluid |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| cGMP | Cyclic guanosine monophosphate |
| FITC | Fluorescein isothiocyanate |
| HBSS | Hank's balanced salt solution |
| IBMX | 3-isobutyl-1-methylxanthine |
| MFI | Mean fluorescence intensity |
| PAF | Platelet activating factor |
| PDE | Phosphodiesterase |
| RAO | Recurrent airway obstruction |

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P-selectin (CD62P), and in vitro studies[6,7] of platelet-neutrophil aggregate formation have revealed a requirement for P-selectin. Little is known in any species about the role of PDE in regulating expression of activation-dependent surface markers on platelets or heterotypic aggregate formation. However, regulation by PDE of P- and E-selectin expression on human endothelial cells and CD11b on human neutrophils has been reported.[8,9] Phosphodiesterase 3 is involved in regulating equine platelet aggregation and adhesion in vitro.[8,10]

Differences in PDE have been identified in lymphocytes of horses with RAO, a chronic inflammatory lung condition in which susceptible horses develop clinical signs following inhalation of organic stable dust.[11] Thus, a selective PDE3 inhibitor causes a significantly greater reduction in PDE activity in lymphocytes from RAO-affected horses without clinical signs, compared with cells from control horses. Exposure to antigen is associated with a significant decrease in the inhibitory effects of a PDE5 inhibitor, as well as a reduction in total cGMP-hydrolyzing activity.[12,13] Altered sensitivity to inhibition by PDE3 and PDE4 selective inhibitors has been detected in lymphocytes and monocytes from allergic human patients with asthma or atopic dermatitis, and an increase in total cAMP-hydrolyzing PDE activity has also been observed.[12,14–16] Furthermore, differential expression of the different isoforms of PDE4 has been described in circulating cells and lung cells from human patients with chronic obstructive pulmonary disease.[17] These data suggest that altered PDE homeostasis occurs in inflammatory lung and skin diseases in humans and horses.

The first objective of the study reported here was to further investigate the regulatory role of PDE4 in stimulus-induced platelet CD41/61 and CD62P expression and in heterotypic aggregate formation in vitro. The second objective was to determine whether, as in equine lymphocytes but not in equine neutrophils, there were differences in the activity or regulation of equine platelet PDE in RAO-affected horses with or without clinical signs (ie, before and after an antigen challenge), a condition in which activation of circulating platelets has been observed 24 hours after exposure to a short antigen challenge.[18]

## Materials and Methods

**Horses—**The study was conducted under a Home Office license approved by The Royal Veterinary College Ethics and Welfare Committee. Seven healthy horses (3 Welsh-cross ponies and 4 Thoroughbreds) were used for in vitro experiments. Five New Forest ponies and a Thoroughbred-cross horse, all with a history of RAO (RAO-affected horses; mean ± SD age, 18 ± 5.8 years [range, 10 to 24 years]), were used in the antigen challenge study. Six healthy ponies (the 3 Welsh-cross ponies used for the in vitro experiments and 3 healthy New Forest ponies that had not been used in the in vitro experiments) were used as control horses. Mean ± SD age of the healthy control horses was 18.5 ± 2.8 years (range, 15 to 21 years). For convenience, the term horse was used in reference to horses and ponies.

All of the RAO horses, but none of the control horses, had previously reacted to a hay and straw challenge with clinical signs of respiratory compromise (ie, increased respiratory rate or effort, nasal discharge, or cough), an increase in pleural pressure, and airway neutrophilia as determined via BALF analysis; no clinical signs were observed when these horses remained on pasture. These findings are consistent with a diagnosis of RAO. All horses were kept at pasture throughout the study period and had not been exposed to hay and straw for at least 1 month prior to the start of the antigen challenge. All horses were vaccinated against tetanus annually and dewormed regularly.

**Isolation of equine platelets—**Blood (100 mL) was collected into plastic tubes containing 0.4M EDTA via jugular venipuncture, and platelets and neutrophils were isolated as described.[18] For flow cytometric studies, blood was collected into a 60-mL syringe containing 3.8% sodium citrate (1 part citrate:9 parts blood) after the first 10 mL of blood had been discarded. Erythrocytes were allowed to sediment for 30 minutes, and the platelet- and leukocyte-rich plasma was collected and centrifuged at 350 $\times$ g for 10 minutes at 4°C (22°C for flow cytometric studies). The platelet-rich plasma was collected and centrifuged at 1,200 $\times$ g for 15 minutes at 4°C (22°C for flow cytometric studies), the supernatant was removed, and platelets were washed twice in 2 mL of calcium- and magnesium ($Ca^{2+}$-$Mg^{2+}$)-free HBSS (1:10 dilution of 10 $\times$ $Ca^{2+}$-$Mg^{2+}$–free HBSS containing 10 mmol/L 4-[2-hydroxyethyl]-1-piperazine ethanesulphonic acid and 0.1% BSA; pH, 7.4). Platelets for flow cytometric studies were not washed. Platelets were resuspended in HBSS, counted by use of a hemacytometer, and suspended at the desired concentrations by use of $Ca^{2+}$-$Mg^{2+}$–containing HBSS (HBSS plus $Ca^{2+}$-$Mg^{2+}$; 1:10 dilution of $Ca^{2+}$-$Mg^{2+}$ containing 10 $\times$ HBSS with 10 mmol/L 4-[2-hydroxyethyl]-1-piperazine ethanesulphonic acid, 4 mmol/L $NaHCO_3$, and 0.1% BSA; final concentration of $Ca^{2+}$-$Mg^{2+}$, 1.2mM and 1mM, respectively). Leukocyte contamination of the platelet preparation was generally < 0.1% (n = 5) as determined via hemacytometer. For determination of PDE activity, platelets (2.5 $\times$ $10^8$/mL) were lysed in a modified lysis buffer that contained (final concentrations) leupeptin (10 µg/mL), Tris-HCl (20mM), $MgCl_2$ (2mM), dithiothreitol (1mM), EDTA (5mM), benzamidine (1.3mM), sucrose (0.25mM), Nα-p-tosyl-L-lysine chloromethyl ketone (20µM), and Triton (1%). Platelet lysates were stored at –80°C and thawed immediately before measurement of PDE activity.

**Isolation of equine neutrophils—**Neutrophils were separated by use of a colloidal silica[a] gradient as described.[18,19] Cell-free plasma for the gradient was prepared from 20 mL of each blood sample via centrifugation (1,400 $\times$ g for 10 minutes at 4°C) and used to prepare the 60% and 80% solutions. The platelet leukocyte-rich plasma was prepared as described, removed, and centrifuged (350 $\times$ g for 10 minutes at 4°C). The cell pellet was resuspended in approximately 15 mL of plasma, layered onto a 60%:80% colloidal silica[a]:plasma gradient (pH, 7.4), and centrifuged (387 $\times$ g for 15 minutes at 4°C). Neutrophils were gently aspirated from the 60%:80% interface with a blunt 18-gauge needle and suspended in 50 mL of HBSS. Following centrifugation



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at 350 × g for 5 minutes at 4°C, the cells were washed twice in HBSS before being resuspended in 1 mL of HBSS and $Ca^{2+}$-$Mg^{2+}$ and counted by use of a hemacytometer. Cells were suspended for use in HBSS and $Ca^{2+}$-$Mg^{2+}$ at a final concentration of $10^6$ cells/mL.

**Influence of cAMP on platelet activation–dependent surface marker expression and platelet-neutrophil aggregate formation—**Platelets were resuspended at a concentration of $5 \times 10^7$ platelets/mL in HBSS and $Ca^{2+}$-$Mg^{2+}$, and the influence of a PDE3 inhibitor (trequinsin[b]), a nonspecific PDE inhibitor (IBMX[b]), and an adenylyl cyclase activator (forskolin[b]) on thrombin (0.1 U/mL)-induced changes in expression of CD41/61 and P-selectin (CD62P) and PAF ($10^{-8}$M)-induced platelet-neutrophil aggregate formation (ratio, 50:1) was determined by use of purified cell populations. The effect of inhibiting PDE5 on these functional responses was not investigated because previous studies[3,20] of equine platelet adhesion and aggregation that used the selective inhibitor zaprinast[b] did not indicate a regulatory role for this isoenzyme. The stimuli chosen were used at concentrations known to induce a substantial functional response in these assays.[21]

Cell stimulation, fixation, and staining were conducted according to the European Working Group Consensus Protocol for flow cytometric characterization of platelet function,[10] as described.[21] In brief, platelets were incubated with trequinsin ($10^{-5}$M), IBMX ($10^{-3}$M), forskolin ($10^{-4}$M), or dimethylsulfoxide (0.1%) for 15 minutes at 37°C prior to the addition of thrombin, PAF, or their diluents (PBS solution containing 0.25% or 0.1% BSA, respectively). Cells were then incubated for 5 minutes (for cell surface activation marker expression) or 10 minutes (for platelet-neutrophil aggregate formation) as described by Segura et al[22] and Peters et al,[23] and the reaction was stopped by addition of paraformaldehyde (0.3%). Platelet and neutrophil suspensions (50 µL) were incubated with 10 µL of FITC-conjugated monoclonal mouse anti-sheep CD41/61[c] or monoclonal mouse anti-human CD62P[d] antibody for 15 minutes at 22°C in the dark. Negative control samples were incubated with an FITC-conjugated isotype-matched antibody.[c]

Analysis of cell surface activation marker expression was performed by use of a flow cytometer[e] and appropriate analytic software.[f] Platelet and neutrophil populations were identified in the same window by use of forward side scatter and side angle scatter (logarithym setting), and 10,000 events were counted. A gate was set to display the MFI of the entire cell population of interest. The percentage of fluorescent cells greater than the negative control value was determined by setting a second gate to display the population of cells exceeding the fluorescence of negative control cells. To standardize the gate setting, the second gate was set to contain 1% of negative control cells.[10] The percentage of fluorescent cells and MFI greater than the negative control value was then determined. Because platelets constitutively express CD41/61 (mean ± SEM, 97.8 ± 0.7% positive cells; n = 7 horses), only the MFI was recorded for this antibody.[10]

To measure platelet-neutrophil aggregates, a gate was set around the neutrophil population, determined by analysis of isolated neutrophils (purity, 97% to 99%; n = 10 horses), and CD41/61 positive events in this gate, representing platelets attached to neutrophils, were counted as described.[6,7] When activated platelets extended into the neutrophil gate, thereby interfering with accurate determination of aggregate formation, data were displayed as compensated FITC (x-axis) versus forward side scatter (y-axis), as described.[21]

**Effect of antigen challenge on platelet PDE activity and sensitivity of PDE3 and PDE5 to inhibition—**Blood samples for measurement of platelet PDE were obtained from 6 RAO and 6 control horses before and after the start of an antigen challenge that was performed as part of a larger study, the details of which are described elsewhere.[21] Measurement of pleural pressure and BALF cytologic findings, as described,[24,25] revealed no evidence of respiratory dysfunction or airway inflammation in either group of horses prior to the challenge. These measurements, together with collection of a prechallenge blood sample, were conducted 48 hours in advance of the challenge to minimize the effect of the BALF collection procedure on subsequent sampling.[26] On the day of the challenge, each horse was placed in a closed box stall containing a standard amount of poor-quality hay and straw for 7 hours, after which the horse was turned out into pasture. The hay and straw were shaken hourly to optimize exposure to dust particles, and dust exposure was quantified by use of a pump[g] calibrated to provide suction at 2.0 L/min. The pump was attached to the animal's head collar with the sampling device positioned close to a nostril. Five hours, 10 hours, and 24 hours after the start of the challenge, further blood samples were obtained, and pleural pressure measurements were recorded at 5 hours and 24 hours. A second sample of BALF was also collected at 24 hours. The presence of RAO was confirmed by observation of the typical clinical signs; observation of an increase in transesophageal pressure at 5 hours, 24 hours, or both; and detection of airway neutrophilia via BALF cytologic examination at 24 hours. Platelet PDE activity was determined at 0 hours, 5 hours, 10 hours, and 24 hours.

**Determination of PDE activity in equine platelets—**Platelet PDE activity was measured by use of a 2-step radioactive assay as described.[3] In brief, 1 µL of either tritium-cAMP or tritium-cGMP was added to a mixture containing 50 µL of platelet lysate or lysis buffer, assay buffer (pH, 8), and the PDE inhibitor–adenylyl cyclase activator or dimethylsulfoxide (0.1%). Tubes containing tritiated substrate alone were also included in each assay. Following incubation at 30°C for 25 minutes, snake venom (*Crotalus atrox* [0.1 mg/kg])[h] was added, and after a 10-minute incubation, duplicate samples were eluted through ion exchange resin[i] and the eluate was counted in a beta counter.[j] The protein content of each sample was determined by use of a colorimetric method,[27] and PDE activity was calculated (pmol/min/mg of protein). Concentration response curves for trequinsin and zaprinast were established by use of platelets from healthy horses and RAO-affected horses (6/group for trequinsin and 4/group for zaprinast [insufficient protein was obtained from 2 horses]). Samples obtained at each time point from 1 RAO-af-



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Table 1—Mean ± SEM values for platelet surface markers and platelet-neutrophil aggregate formation in equine platelets treated with PDE inhibitors or forskolin. Results are expressed as the percentage of positive cells (%) and in arbitrary units of MFI of the entire cell population. *Indicates significant ($P \leq 0.05$) change, compared with stimulus alone.

| Cell type | Surface marker/ cell aggregates | Units | Isotype-matched control | Medium | Stimulus | Trequinsin ($10^{-5}$M) | IBMX ($10^{-4}$M) | Forskolin ($10^{-3}$M) |
|---|---|---|---|---|---|---|---|---|
| Platelets | CD41/61 | (MFI) | 7 ± 1 | 4,112 ± 732 | 5,030 ± 519 | 3,022 ± 210* | 2,977 ± 105* | 3,199 ± 199* |
| Platelets | CD62P | (%) | 1 | 25 ± 2 | 33 ± 1 | 23 ± 1* | 23 ± 2* | 24 ± 2* |
| Platelets | CD62P | (MFI) | 7 ± 1 | 29 ± 5 | 45 ± 4 | 26 ± 1* | 25 ± 1* | 27 ± 1* |
| Neutrophils-platelets | N-P aggr | (%) | 1 | 59 ± 1 | 81 ± 2 | 71 ± 1* | 82 ± 2 | 82 ± 3 |
| Neutrophils-platelets | N-P aggr | (MFI) | 49 ± 2 | 231 ± 6 | 1,625 ± 262 | 853 ± 295* | 1,887 ± 388 | 1,742 ± 337 |

N-P aggr = Neutrophil-platelet aggregations.

fected and 1 control horse were analyzed at the same time in duplicate to minimize variability within and between groups. The effects of trequinsin were examined because of evidence that PDE3 plays a regulatory role in equine platelet activation. The effects of zaprinast were examined in view of the reduction in total lymphocyte cGMP-hydrolyzing activity and the amount of inhibition caused by zaprinast in cells from RAO-affected horses, compared with that for control horses that had been observed following antigen challenge.[13]

**Statistical analysis—**Data are expressed as mean ± SEM values. The effects of PDE inhibition and adenylyl cyclase activation were analyzed by use of 1-way ANOVA followed by the Dunnett multiple comparisons test. Changes in platelet PDE activity and the sensitivity of PDE3 and PDE5 to inhibition after the start of the antigen challenge were analyzed by use of a 3-way hierarchical ANOVA with the Dunnett multiple comparisons test following logarithmic transformation of the data. Analysis of the level of dust exposure in RAO-affected and control horses and the effects of antigen challenge on pleural pressure and BALF cytologic findings within each group is reported elsewhere.[21] Statistical analysis software[k] was used for all analyses. A value of $P \leq 0.05$ was considered significant.

## Results

**Influence of cAMP on expression of platelet activation–dependent surface markers and platelet-neutrophil aggregate formation—**The increases in MFI of platelet CD41/61 and CD62P induced by thrombin were significantly inhibited by trequinsin ($P = 0.002$), IBMX ($P = 0.001$ and $P = 0.002$, respectively), and forskolin ($P = 0.003$ and $P = 0.004$, respectively). The percentage of CD62P positive cells was also significantly ($P = 0.002$) reduced by all 3 compounds (**Table 1**). In contrast, PAF-induced platelet-neutrophil aggregate formation and the MFI within the neutrophil gate were significantly ($P = 0.014$ and $P = 0.015$, respectively) inhibited only by trequinsin.

**Effect of antigen challenge on platelet PDE activity and sensitivity of PDE3 and PDE5 to inhibition—**Antigen challenge caused significant increases in pleural pressure and the percentage of BALF neutrophils in RAO-affected, but not control, horses.[21] No differences were observed in total platelet PDE activity or sensitivity of platelet PDE isoenzymes to inhibition in RAO-affected horses (without clinical signs) and control horses prior to the start of the antigen challenge. Total cAMP-dependent PDE activity in

Table 2—Mean ± SEM values for cAMP- and cGMP-hydrolyzing activity in platelet lysates in response to a 7-hour natural antigen challenge in horses (n = 6 for cAMP and 4 for cGMP) with RAO and control horses before (0 hour), during (5 hours after initiation of the antigen challenge), and after (10 and 24 hours after initiation) challenge. *Indicates a significant ($P \leq 0.05$) change, compared with value at 0 hours.

| Variable | Time | RAO | Control |
|---|---|---|---|
| cAMP-hydrolyzing activity (pmol/min/mg) | 0 h | 5.6 ± 2.6 | 6.9 ± 4.0 |
| | 5 h | 6.2 ± 1.7 | 5.6 ± 1.1 |
| | 10 h | 5.6 ± 2.1 | 4.3 ± 1.1 |
| | 24 h | 4.2 ± 1.8* | 4.3 ± 1.0 |
| cGMP-hydrolyzing activity (pmol/min/mg) | 0 h | 56.0 ± 10.9 | 81.0 ± 27.2 |
| | 5 h | 47.6 ± 8.8* | 56.5 ± 7.9 |
| | 10 h | 43.3 ± 7.2* | 66.9 ± 15.1 |
| | 24 h | 32.0 ± 6.0* | 39.3 ± 3.8* |

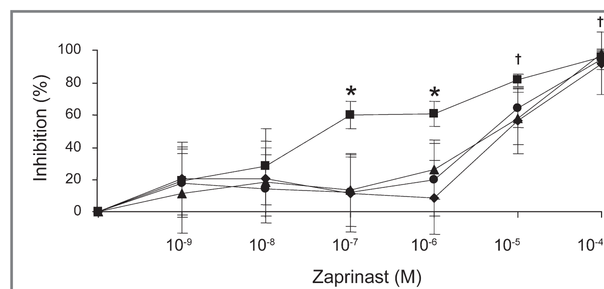


Figure 1—Mean ± SEM percentage inhibition of cGMP PDE activity at 0 (diamonds), 5 (squares), 10 (triangles), and 24 (circles) hours after administration of zaprinast, compared with total PDE activity in the presence of vehicle alone, in 4 RAO-affected horses. *Significant ($P = 0.01$) inhibition at 5 hours. †Significant ($P \leq 0.001$) inhibition at 5 hours. Significant ($P < 0.05$) inhibition at all other time points occurred with concentrations $\geq 10^{-5}$M.

platelets was significantly ($P = 0.02$) lower in the RAO-affected group at 24 hours, compared with the prechallenge value. However, the reduction seemed unlikely to be associated with development of airway obstruction, indicated by increased pleural pressure, because there was a similar numeric reduction in cAMP-hydrolyzing activity in the control group, although the decrease did not reach significance (**Table 2**). Total cGMP-hydrolyzing activity was also decreased in both groups at 24 hours, compared with values at 0 hours ($P < 0.001$ and $P = 0.001$ for RAO-affected and control horses, respectively). In addition, significant decreases were detected at 5 hours ($P = 0.02$) and 10 hours ($P = 0.017$) in RAO-affected horses.

In contrast to the changes in total PDE activity evident in platelets from both groups of horses, an increase in the sensitivity of platelet PDE5 to inhibition by zaprinast was observed only in the RAO-affected horses at 5 hours. At this time, significant ($P = 0.01$) inhibition was obtained at a zap-



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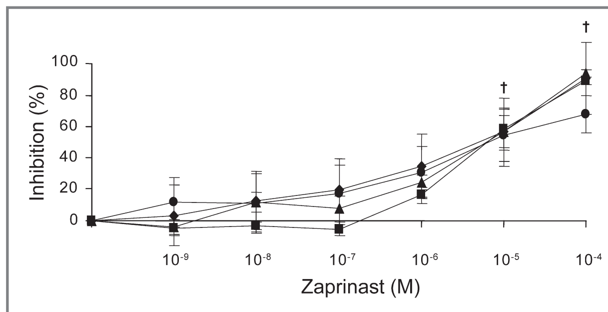


Figure 2—Mean ± SEM percentage inhibition of cGMP PDE activity at 0 (diamonds), 5 (squares), 10 (triangles), and 24 (circles) hours after administration of zaprinast, compared with total PDE activity in the presence of vehicle alone, in 4 healthy control horses. Significant inhibition occurred at all time points for concentrations ≥ $10^{-5}$M. *See* Figure 1 for remainder of key.

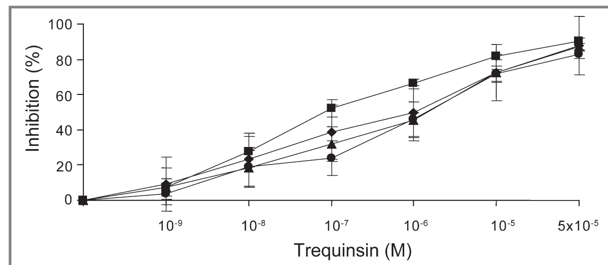


Figure 3—Mean ± SEM percentage inhibition of cAMP PDE activity at 0 (diamonds), 5 (squares), 10 (triangles), and 24 (circles) hours after administration of trequinsin, compared with total PDE activity in the presence of vehicle alone, in 6 ROA-affected horses. Significant ($P \le 0.05$) inhibition was observed at all time points for concentrations ≥ $10^{-7}$M.

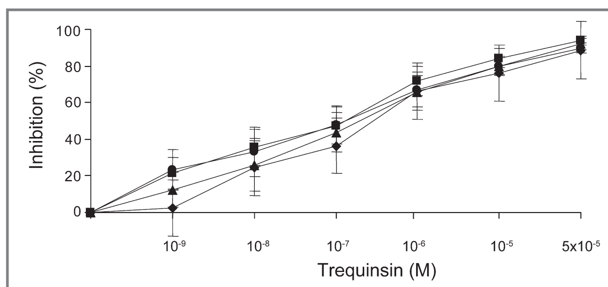


Figure 4—Mean ± SEM percentage inhibition of cAMP PDE activity at 0 (diamonds), 5 (squares), 10 (triangles), and 24 (circles) hours after administration of trequinsin, compared with total PDE activity in the presence of vehicle alone, in 6 healthy control horses. Significant ($P \le 0.05$) inhibition was observed at all time points for concentrations ≥ $10^{-7}$M.

rinast concentration of $10^{-7}$M that was one hundredth of that required at the other time points ($P = 0.003$, $P = 0.02$, and $P = 0.001$ for $10^{-5}$M zaprinast at 0 hours, 10 hours, and 24 hours, respectively; **Figure 1**). Significant inhibition of PDE5 activity in platelets from the control horses was obtained only at a concentration of zaprinast ≥ $10^{-5}$M ($P \le 0.001$), and this did not alter over the period of the challenge (**Figure 2**). The sensitivity of platelet PDE3 activity to inhibition by trequinsin did not change in either group of horses **(Figures 3 and 4)**.

## Discussion

Intracellular cAMP is one of the most important regulators of human platelet activation,[1] and results of in vitro studies of adherence and aggregation suggest that it is also of importance in regulating the functional responses of equine platelets.[3,20] That activation of adenylyl cyclase or inhibition of PDE significantly reduced expression of the surface activation markers CD41/61 and CD62P on equine platelets further suggested that cAMP plays a role in regulating this response. Comparison of CD41/61 expression on platelets pretreated with PDE inhibitors or forskolin with that on unstimulated platelets revealed a numeric decrease in MFI. This could be explained if an increase in surface antigen expression had taken place in the control samples during the 15-minute incubation period, which was prevented by the presence of trequensin, IBMX, or forskolin.

Because CD62P is essential for platelet-neutrophil aggregate formation in horses,[6,7] the finding that PDE3 inhibition decreased heterotypic aggregate formation was not surprising given that trequinsin reduced expression of CD62P. However, in contrast to expression of activation-dependent surface markers, which was reduced to prestimulation values, trequinsin only partially reduced PAF-induced heterotypic aggregate formation. Moreover, neither nonselective inhibition of PDE isoenzymes with IBMX nor activation of adenylyl cyclase with forskolin had an effect. The interplay between intracellular PDE isoenzymes is complex, and interactions between PDE2, PDE3, and PDE5, all of which are present in equine platelets,[3] have been reported in human platelets.[28] For example, the cAMP-hydrolyzing enzyme PDE2 is activated by cGMP, and it has been suggested that nonselective inhibition of PDE3 and PDE5 will increase intracellular cGMP, which in turn activates PDE2.[28] Because this isoenzyme appears to be quantitatively more prominent than PDE3 in equine platelets,[3] a net decrease in intracellular cAMP concentrations could result from nonselective PDE inhibition. However, although this could explain the lack of effect of IBMX, both PAF- and thrombin-induced equine platelet adhesion are significantly reduced by this compound,[18] and another non–isoenzyme selective PDE inhibitor, theophylline, like trequinsin, reduces PAF-induced platelet aggregation. Moreover, it would not explain why forskolin, which increases intracellular cAMP concentrations in a PDE-independent manner, was without effect. The concentration of trequinsin ($10^{-5}$M) was chosen on the basis that it caused almost complete inhibition of equine platelet adherence in a previous study.[3] However, because this concentration is high relative to published concentrations that achieve 50% PDE activity inhibition, and because the concentration that achieves 50% PDE activity inhibition for equine PDE activity has not been established, it is possible that trequinsin reduced platelet-neutrophil aggregate formation in a cAMP-independent manner. Taken together, the data suggest that equine platelet–neutrophil aggregate formation involves cAMP-dependent and -independent mechanisms, as indicated by the partial effect of trequinsin. However, the lack of effect of forskolin and IBMX, despite each compound having reduced stimulus-induced CD62P expression to that of unstimulated platelets, cannot be explained satisfactorily, and further studies will be necessary to delineate the underlying mechanisms that regulate such heterotypic aggregate formation.



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As discussed earlier, compared with cells from healthy horses, differences in PDE activity and its regulation have been described in lymphocytes from RAO-affected horses before and after antigen challenge.[13] In contrast, no differences in neutrophil PDE were observed.[29] In the present study, decreases in total cAMP- and cGMP-hydrolyzing PDE activity were obtained following antigen challenge of horses with RAO. However, because a reduction was also observed in the control group, it seems likely that this occurred as a result of nonspecific airway inflammation caused by high amounts of dust exposure (neutrophil percentage in RAO-affected and control horses increased from 9 ± 3% to 82 ± 0.09% and from 5 ± 0.02% to 32 ± 0.08%, respectively).[21]

It is interesting that the sensitivity of cGMP-hydrolyzing activity to inhibition by the PDE5 selective inhibitor, zaprinast, increased 5 hours after initiation of the antigen challenge in RAO-affected horses. It also contrasts with the report[13] of significantly less inhibition of lymphocyte PDE activity 24 hours after antigen challenge of RAO-affected horses, compared with earlier time points in the same horses and compared with the inhibitory effects in cells from control horses at 24 hours. To the authors' knowledge, there are no other reports of altered PDE5 activity during natural disease processes. Zaprinast was reported to be less effective in causing an increase in intracellular cGMP content in muscle tissue from insulin-resistant rats, compared with that in non–insulin-resistant rats, but the authors suggested that this might be attributable to differences in NO synthase and guanylyl cyclase activity rather than to actual changes in PDE5.[30] Further studies therefore appear warranted before it can be concluded that the regulation of cGMP-hydrolyzing activity by PDE5 is indeed altered in platelets and lymphocytes from RAO-affected horses during antigen challenge.

The study reported here has provided further evidence that PDE3 plays an important role in regulating equine platelet function, suggesting that this isoenzyme could be a potential therapeutic target in conditions associated with platelet activation. There is growing interest in the use of PDE3 inhibitors in a range of human diseases, including intermittent claudication, coronary bypass surgery, and neurologic conditions.[31–33] Theophylline will reduce the clinical signs in RAO-affected horses, although it is a weak, nonselective PDE inhibitor with additional properties that are likely to contribute to its therapeutic benefit, and adverse effects preclude its clinical use.[34] A selective PDE4 inhibitor is of little benefit in RAO.[35] However, with evidence of platelet activation following antigen challenge in RAO and the regulatory role of PDE3, as well as greater inhibition by a PDE3 inhibitor in lymphocytes from RAO-affected horses without clinical signs, evaluation of a PDE3 inhibitor or a combined PDE3-4 inhibitor that would offer bronchodilator and anti-inflammatory activity may be worthy of consideration.[36,37] The changes in platelet and lymphocyte PDE5 inhibition following antigen challenge reported here and elsewhere[13] appear to be associated with RAO. Phosphodiesterase 5 inhibitors can be used to control pulmonary hypertension and inhibit vascular remodeling, and with some limited evidence of such compounds reducing airway dysfunction,[38] examining the effects of a selective PDE5 inhibitor or a compound that combines PDE5 with inhibitory activity on other isoenzymes in RAO may also be warranted.

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a. Percoll polyvinylpyrrolidone-coated colloidal silica, GE Healthcare, Chalfont St Giles, England.
b. Merck Chemicals UK Ltd, Nottingham, England.
c. FITC-conjugated monoclonal mouse anti-sheep CD41/61, AbD Serotec, Oxford, England.
d. FITC-conjugated monoclonal mouse anti-human CD62P, Beckman Coulter Ltd, High Wycombe, England.
e. BD FACSAria, BD Biosciences, San Jose, Calif.
f. FlowJo 7.2.2, Tree Star Inc, Ashland, Ore.
g. Vortex Timer 2, Casella Ltd, Kempston, England.
h. Crotalus atrox venom, Sigma Aldrich, Dorset, England.
i. Dowex, Sigma Aldrich, Dorset, England.
j. 1900 TR, Canberra Packard, Reading, England.
k. SPSS, version 15.0, SPSS Inc, Chicago, Ill.

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