The effect of aspirin on thrombin stimulated platelet adhesion receptor expression and the role of neutrophils

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Aims Aspirin has proven clinical efficacy in limiting the thrombotic complications of atherosclerotic vascular disease but its mechanism of action remains unclear. Recent evidence suggests the anti-platelet action of aspirin may be partly mediated by neutrophil derived nitric oxide (NO). The aim of the study was to determine the effects of aspirin on thrombin-induced platelet expression of the α -granule membrane protein, P-selectin, and the platelet surface glycoprotein required for aggregation, GPIIb-IIIa, and to assess whether this was enhanced by the presence of neutrophils.

Methods Platelet P-selectin and GPIIb-IIIa receptor expression were assessed by flow cytometric analysis of washed platelets stimulated with thrombin (0.025 iu ml^{-1} , sub aggregatory concentration) alone or after pre-incubation with aspirin ($0.05, 0.1, 0.5, 1.0 \text{ mg ml}^{-1}$) either in the presence or absence of neutrophils (100 platelets per neutrophil). NO release was determined by assay of nitrite in the supernatants from parallel samples.

Results In preliminary aggregation studies, aspirin at all concentrations inhibited arachidonic acid but not thrombin-induced platelet aggregation. Similarly, aspirin at all concentrations failed to inhibit thrombin-induced platelet P-selectin or GPIIb-IIIa expression and this was not influenced by the presence of neutrophils. A reduction in P-selectin and GPIIb-IIIa receptor density on non-activated platelets co-incubated with unstimulated neutrophils was associated with NO release from neutrophils, but this was not enhanced by the addition of aspirin.

Conclusions These results confirm that thrombin-induced platelet α -granule release, with consequent P-selectin expression, and platelet GPIIb-IIIa expression, are not affected by aspirin inhibition of cyclo-oxygenase and suggest that the anti-thrombotic efficacy of aspirin *in vivo* may partly depend on other mechanisms. This study did not demonstrate an effect of neutrophils or neutrophil derived NO on aspirin inhibition of platelet adhesion receptor expression.

Keywords: aspirin, platelet, P-selectin, GPIIb-IIIa, neutrophil, nitric oxide

Introduction

Although aspirin is an effective anti-platelet drug for patients with thrombotic vascular disease, its *in vivo* actions remain controversial. Traditionally it has been accepted that aspirin acts via selective inhibition of platelet cyclo-oxygenase and prostanoid production with sparing of endothelial derived prostacyclin [1, 2]. However, recent studies suggest that inhibition of cyclo-oxygenase does not fully explain the clinical benefits of aspirin [3, 4], and there is increasing evidence for the existence of non-platelet-mediated anti-thrombotic effects [5, 6]. Importantly, thrombin and collagen which are major physiological agonists of platelet thrombosis at the site of vascular wall damage, can still induce platelet aggregation *ex vivo*, despite regular aspirin therapy [7, 8].

Platelet-neutrophil interactions are now being recognised

as important modulating factors in thrombotic and inflammatory disease processes [9–12] and the role of aspirin in these intercellular interactions requires clarification. Thrombin-induced platelet aggregation and ATP release *in vitro* have been shown to be inhibited by neutrophils and the inhibitory factor was tentatively identified as nitric oxide (NO) [13]. Unstimulated neutrophils also inhibited platelet serotonin release by a mechanism unrelated to NO, and aspirin enhanced this effect [5]. Independent of neutrophil derived NO, aspirin may also exert anti-platelet effects by inhibiting neutrophil production of superoxide anions [14] which can cause platelet activation [15, 16]. However, a more recent study suggested that aspirin enhances neutrophil inhibition of platelet aggregation by means of a NO related pathway [6]. The discrepancies in these results may relate to different experimental systems and the use of platelet aggregation or serotonin release as markers of platelet activation. However, platelet-neutrophil interactions are complex as emphasised by contradictory data from a variety of investigators showing neutrophils may either enhance or inhibit platelet reactivity [17–23].

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A better understanding of these interactions may be facilitated by flow cytometric assessment of the expression of platelet adhesion receptors following activation [24]. P-selectin is an α -granule membrane protein that is externalised following platelet activation and α -granule release [25]. P-selectin mediates platelet-neutrophil adhesion via interaction with sialyl Lewis^x on the neutrophil counter receptor, P-Selectin Glycoprotein Ligand-1 [26, 27]. P-selectin mediated platelet-neutrophil adhesion promotes intracellular signalling, enhances neutrophil superoxide anion release [28] and facilitates the transcellular metabolism of arachidonic acid with increased production of inflammatory mediators such as thromboxane and leukotriene C₄ [29]. Another platelet glycoprotein, GPIIb-IIIa facilitates fibrinogen binding and the formation of inter-platelet cross bridges, and conformational changes in GPIIb-IIIa represent the final common pathway of platelet activation and aggregation [30, 31]. Assessment of the effects of aspirin on GPIIb-IIIa would therefore, help clarify whether the anti-thrombotic effects of aspirin are mainly mediated by specific inhibition of platelet adhesion or via other mechanisms.

Previous studies have yielded conflicting results regarding the role of aspirin and NO in neutrophil inhibition of platelet activation. Furthermore, it is not clear whether the anti-platelet action of aspirin may be simply to enhance the inhibitory effects of neutrophil derived NO or whether aspirin actually stimulates neutrophil NO release. In view of the current confusion as to the role of aspirin in platelet-neutrophil interactions and its mechanism of action in preventing thrombotic disease, the aims of this study were to assess the effects of aspirin on thrombin induced platelet aggregation, and P-selectin and GPIIb-IIIa expression, and to assess whether neutrophils or neutrophil derived NO modify the effect of aspirin on expression of these platelet adhesion receptors.

Methods

Materials

Lyophilised arachidonic acid (AA, Biodata Corporation, Hatboro, PA, USA) was reconstituted in distilled water according to the manufacturer's instructions and stored at 4° C. Thrombin (Armour Pharmaceuticals Co., Kankakee, IL, USA) was dissolved in isotonic saline and stored at 4° C. Phorbol myristate acetate (PMA, Sigma Chemical Co. St Louis, MO, USA) was dissolved in dimethyl sulphoxide (BDH Chemicals Pty Ltd., Kilsyth VIC, Australia) and stored at -20° C. Before each experiment, PMA, thrombin and AA were further diluted in HEPES buffered Hank's balanced salt solution (HEPES-HBSS) containing 1% bovine serum albumin (BSA, CSL, Parkville, VIC, Australia).

A stock solution of aspirin (Sigma) was prepared by dissolving 30 mg aspirin in 3 ml distilled water together with 45 mg sodium bicarbonate so as to neutralise the acidifying effect of aspirin and to obtain a pH in the physiological range of 7.25-7.5. Studies by other investigators have shown that a pH of less than 6.8 inhibits the sodium-hydrogen pump resulting in the inhibition of adhesion receptor expression [32] independently of any aspirin effect.

Phycoerythrin labelled P-selectin and the isotype control IgG₁FITC/IgG_{2a}PE were obtained from Becton Dickinson (Mountainview, CA, USA). The monoclonal antibody to GPIIb-IIIa (PAC-1) was kindly donated by Dr Sanford Shattil (University of Pennsylvania, Philadelphia, USA). All the antibodies were diluted in HEPES-HBSS/1% BSA.

Subjects

The study population comprised eight normal subjects (four males and four females) aged 31.5 ± 5 years (mean $\pm$ s.d.), with no history of recent infection or trauma. All subjects had abstained from taking aspirin or non-steroidal anti-inflammatory drugs for at least 2 weeks prior to entering the study. Each subject gave informed consent and the study was approved by the Committee for Human Rights of the University of Western Australia.

Blood collection

Blood was taken at 09.00 h with the subjects having fasted overnight and having remained sedentary that morning. To minimise cellular activation, blood was collected atraumatically without use of a tourniquet from an antecubital vein through a 19-gauge butterfly needle. The first 4 ml of blood were discarded and the subsequent 20 ml were collected in two Vacutainer tubes each containing 1.5 ml of acid-citrate dextrose (ACD). A further 40 ml of blood was drawn directly into heparin (1 u ml^{-1}) tubes.

Preparation of washed platelets

Whole blood anti-coagulated with ACD was centrifuged (150 g, 12 min) and the resultant platelet-rich plasma was aspirated and washed with Tyrode's buffer containing 50 ng ml^{-1} PGE₁. After centrifugation (2000 g, 10 min), the platelet pellet was resuspended in Tyrode's buffer containing 2 mM Ca^{2+} . Platelets were diluted to 25×10^6 cells ml^{-1} in HEPES-HBSS/1% BSA and kept at room temperature. For the platelet aggregation assay, washed platelets were diluted 1/10 with platelet poor plasma to replace adhesion factors such as fibrinogen which are necessary for aggregation.

Preparation of neutrophil suspension

Neutrophils were prepared from whole blood anti-coagulated with heparin by centrifugation on discontinuous Percoll gradients. After centrifugation (400 g, 30 min), the plasma and lymphocyte layers were discarded and the neutrophil layer aspirated. The cells were sedimented by centrifugation (400 g, 10 min) and erythrocytes in the pellet were lysed with hypotonic saline. Neutrophils were finally resuspended in HEPES-HBSS containing 2 mM Ca^{2+} and 0.25% BSA, counted and diluted to 25×10^4 cells ml^{-1} . Neutrophil purity and viability were greater than 95% as determined by differential count (Coulter Counter, Coulter, USA), and trypan blue exclusion respectively.

Platelet aggregation assay

Platelet aggregation was measured in a Chronolog lumi-aggregometer (Haverton, PA, USA) at 37°C with constant stirring at 900 rev min⁻¹, and data were normalised for each individual by instrument calibration. For each aggregation test, the required amount of agonist was added to 500 µl of washed platelets in a siliconized glass cuvette. In preliminary experiments, concentration-response curves for AA and thrombin were generated by adding increasing concentrations of these agonists to successive aliquots of resuspended washed platelets to determine the minimum concentration required to achieve the maximum response (MCMR). The amplitude of the aggregation response was measured at a standard time (4 min) after addition of the agonists and expressed as a percentage of the light transmission through autologous platelet poor plasma.

Effect of aspirin on platelet aggregation

The effect of increasing concentrations of aspirin (0.05, 0.5 and 1.0 mg ml⁻¹) on the MCMR of thrombin and AA was determined and compared to an appropriate control. The choice of these aspirin concentrations was based on previous platelet aggregation and adhesion receptor studies and corresponded approximately to the equivalent peak serum concentrations obtained when 30, 300 and 1000 mg aspirin are ingested respectively [8, 33, 34]. For each aggregation test, 50 µl each of increasing concentrations of aspirin solution was added to 450 µl of washed platelets in a siliconized glass cuvette and incubated for 20 min at room temperature. The MCMR of each of the agonists was then added and aggregation was measured as described previously.

Effect of aspirin on P-selectin and GPIIb-IIIa expression on washed platelets

Washed platelets were incubated with varying concentrations of aspirin (0.05, 0.1, 0.5 and 1.0 mg ml⁻¹) for 20 min. Buffer (non-stimulated sample) or 0.025 iu ml⁻¹ of thrombin (a non-aggregatory concentration capable of inducing P-selectin and activated GPIIb-IIIa expression as determined from preliminary optimisation experiments) was then added and incubated for a further 10 min at room temperature. PMA (1.6 µM, based upon data from a previous study [34]) was used as a positive control to determine the maximum possible platelet activation marker expression in each sample.

Effect of aspirin on platelet P-selectin and GPIIb-IIIa expression in the presence of neutrophils

The washed platelet suspension was first incubated with varying concentrations of aspirin (0.05, 0.1, 0.5 and 1.0 mg ml⁻¹) for 20 min. Neutrophils were then added to give a platelet: neutrophil ratio of 100:1 and incubated for a further 10 min. Buffer (unstimulated) or thrombin (0.025 iu ml⁻¹) was then added and incubated for a further 10 min at room temperature. PMA (1.6 µM) was used as a positive control.

Flow cytometry

Following incubation of diluted cell preparations with aspirin and/or agonist, 10 µl of anti-P-selectin or anti-GPIIb-IIIa antibody (diluted 1:40 with HEPES-HBSS/1%BSA) were added. The negative control comprised 10 µl of mouse isotype IgG₁FITC/IgG_{2a}PE. Samples were incubated in the dark at room temperature for 30 min, then fixed with 0.5% (v/v) formaldehyde/BSA and stored at 4°C until analysed on a FACScan flow cytometer (Becton Dickinson). The platelet population was identified by setting analytical gates based on forward and side angle light scatter and confirmed by expression of the selective platelet marker GPIb-FITC. Data were collected in log mode using single parameter analysis of 5000 platelets and binding of the PE or FITC labelled antibody to platelets was quantitated as the percent positive platelets detected. Results were also expressed as mean channel fluorescence (MCF) on a log scale (calculated by subtraction of the negative control MCF from the measured sample MCF) and this is directly proportional to the density of receptors per cell.

Assay of nitrite release by neutrophils

Nitrite, a stable breakdown product of NO in physiological systems, was assayed using the Griess reaction [35]. Supernatants (50 µl) from washed platelets or mixed cell incubations were added to 50 µl of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine, 2.5% phosphoric acid) in triplicate on 96 well plates (Nunc). After incubation at room temperature for 10 min, absorbance at 550 nm was read on a Spectramax 250 plate reader (Molecular Devices, Sydney, Australia). Doubling dilutions of a 100 µM sodium nitrite solution were used to generate a standard curve.

Statistical analysis

Simple descriptive statistics were used to generate mean ($\pm$ s.e.mean) data. For multiple comparisons, analysis of variance was used (PRISM, GraphPad Software, San Diego, CA, USA). Where significant differences were identified ($P < 0.05$), comparisons were made with Tukey's test and the mean differences and associated 95% confidence intervals (CI) are indicated.

Results

Effects of aspirin on platelet aggregation induced by thrombin or arachidonic acid

Incubation of resuspended washed platelets with the lowest concentration of aspirin (0.05 mg ml⁻¹) consistently resulted in maximal inhibition of aggregation induced by 5 mM arachidonic acid (mean difference 65.4%, 95% CI 56.6 to 74.3, $P < 0.001$, Figure 1) and further inhibition of platelet aggregation was not observed with higher concentrations (0.5, 1.0 mg ml⁻¹). In contrast, in all subjects aggregation induced by thrombin (1.0 iu ml⁻¹) was not inhibited following preincubation with any of the aspirin concentrations (0.05, 0.5, 1.0 mg ml⁻¹) ($n = 6$) (Figure 1).

Figure 1 The effect of aspirin on platelet aggregation induced by 1.0 iu ml⁻¹ thrombin (open bars) or 5 mM arachidonic acid (closed bars). Values are means ($\pm$ s.e. mean, $n=6$). For 0.05 mg ml⁻¹ aspirin compared with no aspirin, mean difference = 65.4% (95% CI 56.6 to 74.3, $*P<0.001$).

However, aspirin at the highest concentration did significantly delay aggregation, with the time to maximum aggregation increasing from 0.95 ± 0.12 min in the absence of aspirin to 1.9 ± 0.5 min with 1.0 mg ml⁻¹ of aspirin (mean difference 0.98 min, 95% CI 0.12 to 1.85, $P<0.05$, Figure 2).

Effect of aspirin on P-selectin and GPIIb-IIIa expression on washed platelets

The effect of a range of concentrations of aspirin on thrombin-induced platelet P-selectin and GPIIb-IIIa expression was examined. Thrombin stimulation of platelet P-selectin and GPIIb-IIIa expression was comparable to that of the positive control PMA, indicating adequate activation (Figures 3a, 3b). However relative GPIIb-IIIa receptor density was lower, in keeping with the concentration of thrombin being non-aggregatory (thrombin compared with PMA, mean difference = 88.4 MCF units, 95% CI 30.1 to 146.7, $P<0.001$). Aspirin at varying concentrations (0.05, 0.1, 0.5, 1.0 mg ml⁻¹) failed to significantly inhibit thrombin-induced platelet P-selectin or GPIIb-IIIa expression, as

Figure 2 The effect of aspirin on time to maximum aggregation induced by thrombin (1.0 iu ml⁻¹). Bars indicate means ($n=6$). For 1.0 mg ml⁻¹ aspirin compared with no aspirin, mean difference = 0.98 min (95% CI 0.12 to 1.85, $*P<0.05$).

Figure 3 The percentage of platelets expressing a) P-selectin and b) GPIIb-IIIa in preparations of unstimulated washed platelets (closed bars), platelets stimulated with 1.6 μ M PMA (open bars) and platelets stimulated with 0.025 iu ml⁻¹ thrombin in the presence of increasing concentrations of aspirin (hatched bars). Values are means ($\pm$ s.e. mean, $n=8$). In a) for PMA compared with unstimulated platelets, mean difference = 64.3% (95% CI 47.3 to 81.3, $**P<0.001$) and for thrombin alone compared with unstimulated platelets mean difference = 53.2% (95% CI 36.2 to 70.2, $**P<0.001$). In b) for PMA compared with unstimulated platelets, mean difference = 51.8% (95% CI 17.9 to 85.8, $**P<0.001$) and for thrombin alone compared with unstimulated platelets, mean difference = 48.7% (95% CI 14.8 to 82.7, $*P<0.01$). NS = no significant difference compared with thrombin alone.

determined by the percentage of platelets expressing these adhesion receptors (Figures 3a, 3b). Similarly, thrombin-induced relative receptor densities, as measured by the MCF, were not significantly affected by aspirin.

Effects of co-incubated neutrophils and aspirin on platelet P-selectin and GPIIb-IIIa expression

When unstimulated neutrophils were co-incubated with non-activated platelets, there was a reduction in platelet P-selectin receptor density. However unstimulated neutrophils had no effect on thrombin-induced platelet P-selectin expression or receptor density, even in the presence of aspirin at all concentrations (Figure 4a). Equally, in the presence of unstimulated neutrophils, aspirin did not inhibit thrombin-induced GPIIb-IIIa expression on platelets (Figure 4b), even though the presence of neutrophils induced

Figure 4 The effect of co-incubated neutrophils on the percentage of platelets expressing a) P-selectin, and b) GPIIb-IIIa in preparations of unstimulated platelets (closed bars), platelets stimulated with 1.6 μ M PMA (open bars) and platelets stimulated with 0.025 iu ml⁻¹ thrombin in the presence of increasing concentrations of aspirin (hatched bars). Values are means ($\pm$ s.e. mean, $n=8$). In a) for PMA compared with unstimulated platelets, mean difference = 66.0% (95% CI 48.1 to 83.9, $^{**}P<0.001$) and for thrombin alone compared with unstimulated platelets, mean difference = 52.5% (95% CI 34.6 to 70.4, $^{**}P<0.001$). In b) for PMA compared with unstimulated platelets, mean difference = 52.2% (95% CI 13.3 to 91.1, $^{*}P<0.01$) and for thrombin alone compared with unstimulated platelets, mean difference = 47.0% (95% CI 8.1 to 85.9, $^{*}P<0.01$). NS = no significant difference compared with thrombin alone.

a significant reduction in non-activated platelet GPIIb-IIIa receptor density (mean difference = 34.4 MCF units, 95% CI 5.3 to 63.5, $P<0.05$).

Nitrite production by neutrophils

Supernatants from washed platelets alone contained only trace amounts of nitrite but the nitrite concentration was significantly increased in mixed cell populations (platelets plus neutrophils). Thus for platelets stimulated with thrombin alone compared with platelets plus neutrophils stimulated with thrombin alone, the mean difference was 61.6 pg nitrite/10⁴ cells (95% CI 17.1 to 106, $P<0.01$). However, there was no further increase in nitrite concentration when the mixed cells were incubated with aspirin (Figure 5).

Figure 5 Nitrite concentration in the supernatant from washed platelets alone (open bars) and washed platelets co-incubated with neutrophils (closed bars). Cells were unstimulated or stimulated with thrombin (0.025 iu ml⁻¹) after preincubation with increasing concentrations of aspirin. Values are means ($\pm$ s.e. mean, $n=4$). For unstimulated platelets compared with unstimulated platelets plus neutrophils, mean difference = 68.6 pg nitrite/10⁴ cells (95% CI 24.1 to 113.0, $^{**}P<0.001$). For platelets stimulated with thrombin alone compared with platelets plus neutrophils stimulated with thrombin alone, mean difference = 61.6 pg nitrite/10⁴ cells (95% CI 17.1 to 106, $^{*}P<0.01$) and for platelets stimulated with thrombin and 0.05 mg ml⁻¹ aspirin compared with platelets plus neutrophils stimulated with thrombin and 0.05 mg ml⁻¹ aspirin, mean difference = 53.9 pg nitrite/10⁴ cells (95% CI 9.4 to 98.4, $^{*}P<0.01$).

Discussion

Although aspirin has proven efficacy in the prevention of acute thrombotic events, its mechanism of action remains controversial, as evidenced by its inability to inhibit *in vitro* platelet aggregation to a variety of stimuli [7, 8]. The results from the current study demonstrated that while aspirin at all concentrations abolished aggregation induced by arachidonic acid, it failed to inhibit thrombin-induced aggregation. This is consistent with previous *in vitro* studies [7] and data from comparable studies demonstrating the poor efficacy of aspirin in inhibiting platelet aggregation induced by collagen and platelet activating factor (PAF) [8]. The observed modest delay in thrombin induced aggregation in the presence of aspirin may be due to a reduction in thromboxane production and a consequent delayed amplification of the aggregatory response. Although it would appear that thrombin induced aggregation is not critically dependent on the generation of thromboxane A₂ it is possible that the size of the thrombus may be dependent on its production [36].

In experiments assessing the effect of aspirin on P-selectin expression, thrombin induced a significant increase in the percent of platelets expressing P-selectin and in the receptor density per cell, which is in agreement with previous studies [37, 38]. However, a range of aspirin concentrations failed to inhibit thrombin-induced platelet P-selectin expression even when the platelets were co-incubated with neutrophils. Recent data demonstrating that aspirin does not affect P-selectin expression following stimulation with ADP [32], PAF or collagen [34] together with the findings from this

study, raise questions regarding the dependency of α -granule release on products of arachidonate metabolism [37]. The emerging evidence that platelet activation and release of mitogenic products such as platelet-derived-growth factor (PDGF) from α -granules is not inhibited by aspirin, is further supported clinically by the observation that aspirin and other cyclo-oxygenase inhibitors are unable to prevent atherogenesis or restenosis after coronary angioplasty [39, 40].

Thrombin generation resulting in platelet activation and fibrin formation is thought to be the primary pathogenic mechanism involved in acute thrombosis at the site of plaque rupture [41]. In the assessment of the effect of aspirin on thrombin-induced platelet activated GPIIb-IIIa expression, the results demonstrated that while thrombin induced a significant increase in the percentage of platelets expressing activated GPIIb-IIIa with an associated increase in receptor density, a range of aspirin concentrations failed to inhibit either the increase in the percentage of platelets expressing GPIIb-IIIa or receptor density. These findings suggest that thrombin can induce platelet GPIIb-IIIa expression and subsequent aggregation through a cyclo-oxygenase independent mechanism and this is consistent with the lack of effect of aspirin on thrombin induced aggregation. Furthermore, the lack of effect of aspirin on platelet GPIIb-IIIa expression does not appear to be altered by the presence of neutrophils.

Previous data have demonstrated a platelet anti-aggregating effect of unstimulated neutrophils and it has been proposed this may occur through a nitric oxide cGMP-dependent pathway [6, 42]. Neutrophil-derived nitric oxide could exert anti-thrombotic activity by inhibiting platelet adhesion and aggregation by impairing platelet activation, GPIIb-IIIa expression and/or binding to fibrinogen, P-selectin expression or granule secretion [43]. In the current study, the observation that non-activated platelets co-incubated with unstimulated neutrophils had reduced P-selectin and GPIIb-IIIa receptor density, with an associated increase in NO release in the supernatant, further supports the potential role of neutrophil derived NO as an inhibitor of platelet function. However, the inability of aspirin to inhibit thrombin-induced expression of these receptors or to have an effect on NO release in the mixed cell system, does not support the concept that inhibition of platelet activation *in vitro* may be enhanced by an aspirin effect on neutrophil derived NO. This strongly suggests that aspirin does not stimulate neutrophil production of NO, which is in contrast to the results reported by Lopez-Farre *et al.* [6]. However, whether this may be due to different levels of neutrophil activation (and different levels of NO release) during cell preparation or requires specific neutrophil activation is currently unclear.

The lack of effect of aspirin on *in vitro* thrombin-induced platelet P-selectin and GPIIb-IIIa expression and its inability to stimulate any NO mediated anti-platelet effect in neutrophils contrasts with the potency and effectiveness of aspirin as an anti-thrombotic agent *in vivo* [2]. However, aspirin may have other actions *in vivo*, in addition to inhibiting platelet cyclo-oxygenase. Thus, aspirin has been shown to impair generation of thrombin in clotting blood, possibly by acetylating prothrombin or platelet membrane proteins [44]. Interaction of aspirin with platelet membranes

may also induce changes in protein conformation and membrane fluidity that may impair platelet function [45]. In addition, aspirin has been shown to inhibit neutrophil-endothelial cell attachment *in vitro* by decreasing expression of L-selectin on the neutrophil surface [46]. However, the relevance of these alternative effects of aspirin to its anti-thrombotic efficacy *in vivo* requires further investigation.

It is a well established therapeutic concept that aspirin acts through inhibition of platelet cyclo-oxygenase resulting in reduced thromboxane A₂ formation. However, our findings suggest that depending upon the stimulus, the activation of platelet adhesion receptors, subsequent platelet fibrinogen binding and release of platelet α -granules can continue despite apparent effective inhibition of platelet cyclo-oxygenase. The data do not exclude the possibility that aspirin inhibits the secondary amplifying effect of platelet thromboxane A₂ production but the anti-thrombotic efficacy of aspirin *in vivo* may also depend in part on mechanisms unrelated to the inhibition of platelet cyclo-oxygenase and this warrants further investigation.

We would like to thank the Raine Medical Research Foundation (University of Western Australia) and the R. F. Shaw Foundation (Sir Charles Gairdner Hospital, Perth) for financial support and the Department of Haematology, Sir Charles Gairdner Hospital for technical support.

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(Received 23 June 1997,
accepted 20 February 1998)