Published in final edited form as:

Platelets. 2014 ; 25(1): 1–7. doi:10.3109/09537104.2013.773969.

Natriuretic peptides induce weak VASP phosphorylation at Serine 239 in platelets

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Abstract

Cyclic guanosine-3',5'-monophosphate (cGMP) is the common second messenger for the cardiovascular effects of nitric oxide (NO) and natriuretic peptides (NP; for example, atrial natriuretic peptide [ANP]), which activate soluble and particulate guanylyl cyclases (sGC and pGC), respectively. The role of NO in regulating cGMP and platelet function is well documented, whereas there is little evidence supporting a role for NPs in regulating platelet reactivity. By studying platelet aggregation and secretion in response to a PAR-1 peptide, collagen and ADP, and phosphorylation of the cGMP-dependent protein kinase (PKG) substrate VASP at serine 239, we evaluated the effects of NPs in the absence or presence of the non-selective cGMP and cAMP phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX). Our results show that NPs, possibly through the clearance receptor (natriuretic peptide receptor-C, NPR-C) expressed on platelet membranes, increase VASP phosphorylation but only following PDE inhibition, indicating a small, localised cGMP synthesis. As platelet aggregation and secretion measured under the same conditions were not affected, we conclude that the magnitude of PKG activation achieved by NPs in platelets per se is not sufficient to exert functional inhibition of platelet involvement in haemostasis.

Keywords

Natriuretic peptides; VASP; platelets

Introduction

Under physiological conditions, platelet activation is tightly regulated. However, this is impaired in cardiovascular diseases and can underlie life-threatening thrombosis at sites of atherosclerotic plaques or following ischaemic heart disease. Among the many factors that contribute to dysregulated platelet activation is reduced bioactivity of the platelet inhibitors nitric oxide (NO) and prostacyclin (PGI₂) released from the vascular endothelium. NO activates soluble guanylyl cyclase (sGC), which generates cyclic guanosine-3'-5'-monophosphate (cGMP), leading to protein phosphorylation by the cGMP-specific protein kinase (PKG)[1]. The platelet inhibitory functions of cGMP can be attributed to phosphorylation of a range of PKG substrates, including HSP27, inositol-1,4,5-triphosphosphate (IP₃) receptors, IP₃-associated cGMP kinase substrate (IRAG), myosin light chain kinase, Rap-1b, the TxA₂ receptor and the vasodilator-stimulated phosphoprotein

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Declaration of Interest statement The authors have no conflict of interest to declare.

(VASP), (for review see[2, 3]). Phosphorylated VASP hampers actin filament-mediated reorganisation of the cytoskeleton and inside-out activation of the integrin $\alpha_{IIb}\beta_3$ [4, 5]. Phosphorylation of Ser 157 and Ser 239 is carried out preferentially by PKA and PKG, respectively [6, 7].

cGMP synthesis is also controlled by a second GC enzyme expressed in the plasma membrane, termed particulate GC (pGC). pGC is activated by a family of natriuretic peptides (NPs), which govern systemic vascular resistance, central venous pressure, and cardiac contractility[8]. The principal NPs are atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP)[9]. ANP and BNP are secreted primarily by the atria and ventricles of the heart, respectively, while CNP is secreted by the vascular endothelium. Two pGC subtypes that act as receptors for NPs, termed NP receptors (NPR), have been identified, namely NPR-A and NPR-B[10-12]. NPR-A binds ANP and BNP with equal affinity, whereas CNP appears to be the endogenous ligand for NPR-B. A third receptor, NPR-C, has a similar affinity for all three peptides, but is not linked to pGC. This protein is thought to act as a clearance receptor, removing NPs from the circulation[9]. In addition, NPR-C has also been reported to activate GTP-binding proteins that regulate adenylyl cyclase, phosphoinositide turnover, and vascular function[13, 14].

In contrast to the well-characterised role for the NO-sGC-cGMP pathway in regulating platelet function, the role of NP-pGC-cGMP in platelets is poorly characterised and controversial. The presence of NPR-A on platelet membranes was originally proposed based on ANP binding assays[15]. However, these findings were questioned because of the lack of influence of ANP on cGMP levels in platelets[16] and because it had no significant physiological effect on platelet function[17]. This observation was further supported by data identifying expression of NPR-C, but not NPR-A and NPR-B on platelets, and confirmation of the lack of effect of ANP on intracellular cGMP levels[18]. Despite these findings, Loeb and Gear (1988) reported that ANP increased platelet cGMP levels but that this paradoxically potentiated platelet aggregation[19]. Further, it has been previously reported that CNP and the selective agonist at the platelet clearance receptor, cANF⁴⁻²³ inhibit leukocyte recruitment and platelet-leukocyte interactions illustrating a role for NPR-C in regulating the inflammatory role of platelets[20].

In view of these contrasting observations, we have carried out an extensive investigation of the effects of ANP, BNP, CNP and the selective NPR-C ligand, cANF⁴⁻²³ on platelet function. We show that although NPs and cANF⁴⁻²³ potentiate phosphorylation of the PKG substrate VASP on Ser 239, possibly reflecting localised cGMP synthesis, they have no functional effect on platelet aggregation and dense granule secretion.

Methods

Materials

PAR1 activating peptide (SFLLRN, indicated as PAR1 below) was purchased from Alta Bioscience (Birmingham, UK), collagen from Nycomed (Linz, Austria). Chrono lume and ATP were obtained from Chrono Log Corporation (Manchester, UK). Human ANP (hANP) and CNP were from Millipore (Bucks, UK); rat ANP (rANP) and BNP were from Sigma (Poole, UK) and cANF⁴⁻²³ was from Bachem (Bubendorf, Switzerland). Antibodies were obtained from the following sources: anti-VASP p-ser 239 and anti-VASP p-ser 157 (Cell signalling Technology, Herts, UK); anti-tubulin (Sigma, Poole, UK); anti-rabbit fl-800 and anti-mouse fl-680 (LI-COR Biosciences, Lincoln, NE, USA). PVDF-FL was from Millipore (Bucks, UK). Thrombin, 3-isobutyl-1-methylanthine (IBMX) and S-nitrosoglutathione (GSNO) and other reagents not specified were from Sigma (Poole, UK).

Platelet aggregation and secretion

Human blood was obtained from healthy donors who had given written, informed consent. The investigation conforms to the principles outlined in the Declaration of Helsinki. The studies were approved by the University of Birmingham Ethical Review Committee (ERN_10-0625). All donors denied having taken medication known to influence platelet function (e. g. non steroidal anti-inflammatory drugs, NSAIDs) in the previous 14 days. Blood was drawn into 10 % sodium citrate. Washed platelets were prepared as previously described[21] and resuspended in Tyrode's buffer (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES, 5 mM glucose, 1 mM MgCl₂, pH 7.3) at $2-5 \times 10^8$ /ml.

Platelet aggregation was measured in a lumi-dual aggregometer (model 460VS; Chronolog, Labmedics, Manchester, UK) under continuous stirring at 1200 rpm. The optical density of the platelet suspension was measured against a blank reading of Tyrode's buffer (100%). Chronolume (1: 30) was incubated in the suspension for measurement of ATP secretion from dense granules. Calibration was obtained by addition of 1.2 nmol ATP at the end of the reaction. NPs, IBMX, the GSNO or vehicles were incubated for the stated time before platelet activation with 3-10 μ M PAR1 or 1-3 μ g/ml collagen.

VASP western blot

Platelets were washed as described above and suspended in Tyrode's buffer at 5×10^8 /ml. Stimulations were carried out in a Chrono Log Lumi-dual aggregometer (model 460VS; Chronolog, Labmedics, Manchester, UK) under continuous stirring at 1200 rpm at 37 °C. NPs, IBMX, GSNO or vehicles were incubated for 3 min before lysis with Laemmli sample buffer. Samples were boiled for 5 min and spun for 3 min to remove cellular debris before loading on 10 % gels for SDS-PAGE. Proteins were electrotransferred on PVDF-FL membranes, blocked to prevent non-specific binding, and probed with primary antibodies as stated. Immunodetection was performed with appropriate fluorescent conjugated secondary antibodies and fluorescent bands were visualised in a LI-COR Odyssey infrared scanner (Lincoln, NE, USA) with a laser intensity that avoided band overexposure. Quantitative analysis of band intensity was performed with the Odyssey software according to manufacturer's instructions.

Statistical analysis

Results are presented as one representative experiment of the stated number of independent experiments or in chart form as mean $\pm$ SEM. Differences among groups were evaluated by one way or two way ANOVA test as appropriate with Bonferroni post test. $P < 0.05$ was considered significant.

Results

Natriuretic peptides do not affect platelet aggregation

We have used several NPs (rat ANP, and human, BNP and CNP) and the clearance receptor agonist, cANF⁴⁻²³, at various concentrations (0.1-10 μ M) and incubation times (1-10 min) before inducing platelet aggregation with PAR1 activating peptide (PAR1), which signals through the Gq-coupled PAR1 receptor, and collagen, which signals through the tyrosine kinase-linked collagen receptor, GPVI. As illustrated in Figure 1a, b and 2, neither aggregation nor dense granule secretion induced by intermediate concentrations of PAR1 (10 μ M) and collagen (3 μ g/ml) was significantly affected by ANP, BNP, CNP and cANF⁴⁻²³ (0.1-10 μ M). Aggregation induced by low concentrations of PAR1 (3 μ M) and collagen (1 μ g/ml) was not affected by high concentrations of NPs (Fig 1c). Granule secretion induced by these concentrations of agonists was minimal (PAR1) or null

(collagen), therefore the traces are not shown. Similar results were obtained in ADP-sensitive washed platelets for ADP in the presence of BNP and cANF⁴⁻²³ (data not shown). Other NPs were not used as the buffer used to dissolve the peptide inhibited aggregation to ADP (not shown).

Natriuretic peptides induce VASP phosphorylation at serine 239

To determine whether the NPs induce phosphorylation of the PKG substrate VASP, as a read-out of PKG activity and indirectly of cGMP synthesis, we used quantitative Western blotting for VASP at the site for PKG phosphorylation, Ser239. In addition, we studied phosphorylation at Ser157, preferentially phosphorylated by PKA, in order to exclude any PKA activation. Further, since VASP is a cytoplasmic protein and cGMP synthesis by pGCs occurs at the plasma membrane, we used a low concentration of the non-specific PDE inhibitor, IBMX, to enhance cyclic nucleotide formation. Figure 3a shows a representative blot for VASP phosphorylation at Ser239 and Ser157 in samples treated with NPs in the presence or absence of IBMX. Alone, ANP, BNP or CNP had no significant effect on VASP phosphorylation, but when used in combination with a sub-threshold concentration of IBMX (30 μ M; a concentration which induced weak VASP phosphorylation), BNP and cANF⁴⁻²³ caused a significant increase ($p < 0.01$) in VASP phosphorylation at Ser239 relative to the IBMX-treated samples (Fig 3b). ANP and CNP also caused a synergistic increase in Ser239 phosphorylation in the presence of IBMX in some but not all experiments. These results therefore provide indirect evidence for a small, localised cyclic nucleotide synthesis by NPs. The NO donor, GSNO, was used to stimulate cGMP synthesis through the well-established sGC-cGMP pathway as a positive control for VASP phosphorylation (Fig 3a). IBMX also induces VASP phosphorylation at Ser157, as expected due to its non-specific inhibition of PDEs leading to both cGMP and cAMP elevation. On the other hand, the combined treatment with NPs doesn't result in a synergistic phosphorylation on this site, suggesting that PKA is not activated following treatment with NPs (Fig3a, b).

To determine whether the increased PKG activity (via VASP phosphorylation) obtained following the combined use of NPs and IBMX was reflected by functional changes in platelet aggregation and dense granule secretion, platelets were treated with a subthreshold concentration of IBMX (Fig4a) in combination with NPs and cANF⁴⁻²³. As shown in Figure 4b,c, platelet aggregation and secretion measured in the same conditions were not affected by IBMX and NPs or cANF⁴⁻²³. In contrast, GSNO strongly inhibited platelet aggregation (as expected, by activation of the NO-sGC pathway) (Fig 4b).

It should be noted that 0.1 μ M GSNO does not affect aggregation while 1 μ M abolishes the response to 10 μ M PAR1. Accordingly, VASP phosphorylation is only weakly increased by the lower concentration of NO-donor and strongly enhanced by the higher concentration. Notably, the increase in VASP Ser239 phosphorylation achieved with combinations of NPs and IBMX is only slightly higher than that reached with 0.1 μ M GSNO, indicating that the amount of cGMP produced by NPs or cANF⁴⁻²³ in combination with IBMX or by 0.1 μ M GSNO is not sufficient to achieve functional inhibition.

Discussion

Endogenous mediators released by the intact vascular endothelium such as PGI₂ and NO act both as vasodilators and as platelet inhibitors via the intracellular generation of cyclic nucleotides. Several studies have assessed whether NPs, which act as vasodilators and regulators of cardiac contractility, blood volume and pressure, display similar inhibitory effects on platelets, but reported conflicting data[13, 14, 16, 17]. Moreover, a comprehensive study considering all NPs and cANF⁴⁻²³ in one experimental setting was lacking.

In this study, we have investigated platelet reactivity in the presence of a variety of natriuretic peptides and a specific ligand (cANF⁴⁻²³) for the clearance receptor NPRC which, to date, is the only NP receptor reported to be expressed in platelets[22]. Although NPR-C functions as a clearance receptor for NPs, several studies have reported a range of signalling activities[23, 24]. We tested the effects of NPs and cANF⁴⁻²³ on the major platelet functional responses, aggregation and dense granule release. Our data show that NPs, even at supra-physiological concentrations are unable to affect aggregation and ATP release from dense granules.

The lack of global cellular increase in cGMP synthesis in platelets in response to NPs has been reported[18]. However, a small local increase might still occur at the membrane and affect spatial-restricted (proximate) responses, without causing an increase in the total cGMP pool. Indeed, recent studies support the notion of a compartmentalised cyclic nucleotide signalling in platelets[25, 26] as well as in other cells, such as cardiomyocytes[27]. Such signalling, based on strictly regulated localised synthesis and degradation of cyclic nucleotides, might have been overlooked in previous work with whole cell cyclic nucleotide measurements. In order to highlight any localised cyclic nucleotide signalling, we inhibited PDE-dependent degradation and evaluated the phosphorylation status of the PKG substrate VASP at Ser239. Our results demonstrate that NPs and cANF⁴⁻²³ induce VASP phosphorylation in the presence of PDE inhibition, thus suggesting that they cause a localised generation of cGMP-PKG activation that is counteracted by PDEs. To our knowledge, this is the first evidence that NPs and cANF⁴⁻²³ induce phosphorylation of VASP Ser239 in platelets. The results with cANF⁴⁻²³ is of particular interest given that it has not been previously shown to activate soluble or particulate GCs.

Our experiments with the NO-donor GSNO, used as a positive control for cGMPPKG signalling in platelets, demonstrate that the amount of VASP phosphorylation induced by combinations of IBMX and NPs is only slightly higher than that achieved by a concentration of GSNO (0.1 μ M) which is not able to inhibit functional responses. These results are in line with the lack of functional effect of NPs used in the presence of PDE inhibition.

Although the existence of a signalling pathway dependent on NPs in platelets is undoubtedly novel and interesting per se, the lack of functional consequences, at least on the responses considered in this work, suggest that NPs do not play a major physiological role in the regulation of platelet function. Nevertheless, it is possible that they play an important role in synergy with other platelet agonists. This action could be particularly significant in combination with the anti-inflammatory action of the clearance receptor which inhibits leukocyte recruitment and platelet-leukocyte interactions[20]. Indeed, in this regard studies in mice with an endothelial-specific deletion of CNP have revealed these animals to have increased platelet P-selectin expression and greater numbers of platelet-leukocyte aggregates, intimating CNP, at least, is likely to influence platelet function *in vivo*[28].

Acknowledgments

We are grateful to Prof. Adrian Hobbs for very useful discussion.

This work was supported by the British Heart Foundation (BHF PG/12/22/29485). SPW holds a BHF chair.

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Figure 1. Natriuretic peptides do not affect platelet aggregation and secretion

The effects of NPs were studied with light transmission aggregometry (LTA) and measurement of dense granule secretion. 2×10^8 /ml washed platelets containing chrono lume were incubated with NPs (10 μ M hANP, 10 μ M rANP, 10 μ M BNP, or 3 μ M CNP) for 3 min before addition of 10 μ M PAR1 (a), 3 μ g/ml collagen (b) or 3 μ M PAR1 and 1 μ g/ml collagen (c). Representative traces of four independent experiments are shown.

Figure 2. Stimulation of NPR-C does not affect platelet aggregation and secretion

The effects of the stimulation of NPR-C was evaluated with LTA and measurement of dense granule secretion using cANF⁴⁻²³. 2×10^8 /ml washed platelets containing chrono lume were incubated with increasing concentrations of cANF⁴⁻²³ (0.1-10 μM) for 1-10 min before addition of 10 μM PAR1 (a) or 3 μg/ml collagen (b). Percentage of aggregation reached after 4 min was calculated as percentage of control (100%, dotted line) and plotted as mean $\pm$ SEM of the different experiment performed (n=4) (a, b). Representative PAR1 (c) and collagen (d) aggregation traces are shown (cANF⁴⁻²³ incubated for 3 min).

Figure 3. NPs induce VASPser239 phosphorylation in the presence of IBMX

The effect of NPs on VASP phosphorylation at serine 239 and 157 was assessed in the presence or in the absence of IBMX. 5×10^8 /ml washed platelets were incubated with IBMX (30-100 μ M) and/or NPs (10 μ M hANP, μ M rANP, μ M BNP, 3 μ M CNP, 10 μ M cANF⁴⁻²³) or vehicles for 3 min. GSNO (0.1-1 μ M) was incubated 3 min as a positive control for VASP phosphorylation. Platelets were lysed by addition of 5x Laemmli sample buffer and subjected to SDS-PAGE and Western blot analysis. Blots were incubated with primary antibodies (anti VASP p-Ser 239 antibody or anti VASP p-Ser 157 1: 1000 and anti-tubulin antibody 1: 5000) followed by incubation with fluorescent secondary antibodies (anti rabbit or anti mouse 1: 10000). Fluorescent bands were detected by Odyssey Infrared scanner. Representative blots of five identical experiments are shown (a). Quantitative analysis of band intensity was performed with Odyssey software. Integrated intensities (AU) were normalised as fold increase over basal and reported as mean $\pm$ SEM of the different experiment performed (n=5). Panel b and c show quantitative analysis for phosphorylation at Ser 239 and 157 respectively. Differences among groups were evaluated by two way ANOVA and Bonferroni post test (** p<0.01, non significant (ns)).

Figure 4. NPs and IBMX do not synergise to inhibit platelet aggregation or secretion

The effects of the combination of NPs and a subthreshold concentration of IBMX were studied with light transmission aggregometry (LTA) and measurement of dense granule secretion. 2×10^8 /ml washed platelets containing chrono lume were incubated with IBMX at increasing concentrations or GSNO as a positive control for platelet inhibition (a). NPs (10 μ M hANP, 10 μ M rANP, 10 μ M BNP, 3 μ M CNP or 10 μ M cANF⁴⁻²³) were incubated for 3 min in the presence of 30 μ M IBMX before addition of 10 μ M PAR1 (b, c).

Representative traces of four independent experiments are shown (b). Percentage of aggregation reached after 4 min was calculated as percentage of control and plotted as mean $\pm$ SEM of the different experiment performed (c).