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The C-terminal peptide of thrombospondin-1 stimulates distinct signaling pathways but induces an activation-independent agglutination of platelets and other cells

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Abstract A peptide from the C-terminal domain of thrombospondin-1 (4N1-1) has been proposed to stimulate platelet aggregation by a novel mechanism involving both an activation-independent agglutination and an activation-dependent, glycoprotein (GP) IIb/IIIa-mediated aggregation which involves GPVI signaling but does not involve CD47. The present study demonstrates that 4N1-1 stimulated a different pattern of signal transduction pathways than the GPVI agonist convulxin. Furthermore, 4N1-1-induced platelet aggregation was activation-independent and not dependent on GPVI or GPIIb/IIIa. Interestingly, 4N1-1 also stimulated activation-independent agglutination of different megakaryocytic and non-megakaryocytic cells. 4N1-1-induced cell agglutination but not platelet signaling was inhibited by anti-CD47 antibodies.

Key words: Platelet; Thrombospondin-1; 4N1-1; Signaling

1. Introduction

Thrombospondin-1 is a multidomain glycoprotein, which is stored in the α-granules of platelets [1]. Upon platelet activation it becomes secreted and binds to the cell surface where it contributes to the stabilization of platelet aggregates [2]. 4N1-1 (H-Arg-Phe-Tyr-Val-Val-Met-Trp-Lys-OH) is a peptide from the C-terminal cell binding domain of thrombospondin-1. In contrast to the full-length protein, 4N1-1 was reported to induce different signaling events. On immobilized matrices it was shown to cause platelet spreading and aggregation in a tyrosine kinase-, phosphatidylinositol 3-kinase- and protein kinase C-dependent fashion [3–5]. The receptor mediating these effects appears to be the integrin-associated protein CD47, since a direct binding for 4N1-1 could be demonstrated [3] and 4N1-1-induced platelet aggregation on immobilized collagen was abolished in CD47−/− mice [5]. These data collectively suggested that CD47 mediates the activating effects of 4N1-1 on platelets adhering to immobilized matrices.

However, a recent study has demonstrated that 4N1-1-induced aggregation of stirred platelets occurred in CD47−/− mice [6]. In addition, 4N1-1 has been proposed to induce both an activation-independent platelet agglutination and an activation-dependent, glycoprotein (GP) IIb/IIIa-mediated platelet aggregation. 4N1-1 stimulated a rapid tyrosine phosphorylation of the FcRγ chain, Syk, SLP-76, and phospholipase Cγ2, thus indicating that 4N1-1 signals through a pathway similar to that of GPVI agonists, such as convulxin [7,8]. In addition, the phosphorylated FcRγ chain in platelets stimulated with 4N1-1 was shown to coimmunoprecipitate with GPVI. However, it is not clear if GPVI is directly activated by 4N1-1 in platelets. Thus, there is controversy about the mechanisms of 4N1-1-induced platelet aggregation.

The present study investigated the effects of 4N1-1 as compared to thrombin and convulxin on several signaling pathways and the effects of platelet inhibitors on 4N1-1-induced platelet aggregation. We demonstrate that 4N1-1 stimulates distinct signaling pathways in platelets different from those induced by the GPVI-specific agonist convulxin and that 4N1-1-induced aggregation is not altered in GPVI-deficient mouse platelets. Furthermore, we show that 4N1-1-induced aggregation occurs entirely independently of GPIIb/IIIa receptors, is not cell type-specific, and is not dependent on cellular activation. CD47 may mediate cell agglutination induced by 4N1-1.

2. Materials and methods

2.1. Materials

H-Arg-Phe-Tyr-Val-Val-Met-Trp-Lys-OH (4N1-1) was from Bachem Biochemica (Heidelberg, Germany). Phospho-specific antibodies against Akt (Ser^{473} and Thr^{308}), p38 mitogen-activated protein (MAP) kinase and extracellular signal-regulated kinases (ERK)-1 and 2 were from Cell Signaling Technology (Beverly, MA, USA). Anti-CD47 antibodies (clone B6H12) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorescein isothiocyanate-conjugated antibodies against CD62P were from Coulter Immunotech (Marseille, France). PP2 was from Calbiochem-Novabiochem (San Diego, CA, USA). Iloprost was from Schering (Berlin, Germany). Abciximab was from Centocor (Leiden, The Netherlands). Eptifibatide was from Essex Pharma (Munich, Germany). Tirofiban was from Merck Sharp and Dohme (Haar, Germany). Oregon-Green fibrinogen was from Molecular Probes (Eugene, OR, USA). Convulxin was from Pentapharm (Basel, Switzerland). α-Thrombin was a gift from Dr. Stürzebecher (Erfurt, Germany). All other chemicals were from Sigma (Deisenhofen, Germany).

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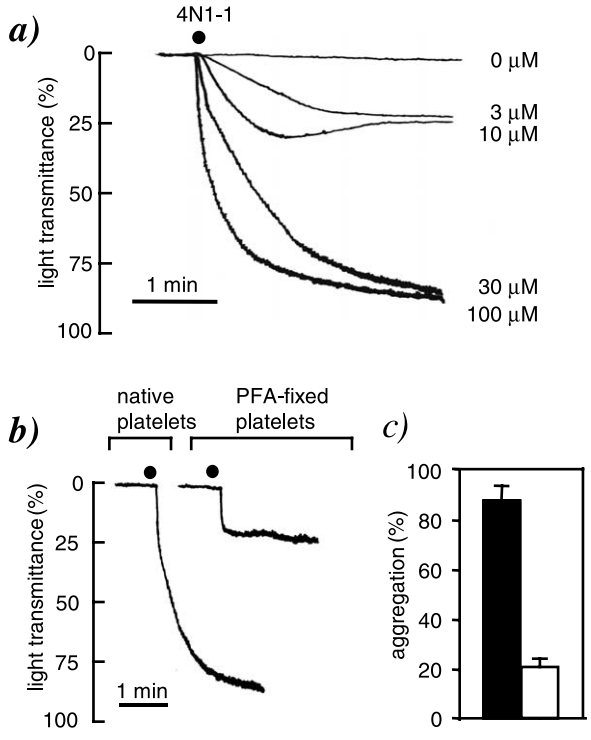

Fig. 1. Effects of 4N1-1 on aggregation of native and paraformaldehyde (PFA, 1%)-fixed platelets. a: Original tracings, demonstrating a concentration-dependent effect of 4N1-1. b: Original tracings demonstrating the effects of 4N1-1 on aggregation of native as compared to PFA-fixed platelets. c: Quantitative comparison of the effects of 4N1-1 on aggregation of native (closed bar) as compared to PFA-fixed platelets (open bar) (means ± S.E.M., $n = 6$).

2.2. Preparation of washed platelets and measurement of platelet aggregation and cell agglutination

Platelet-rich plasma was prepared from buffy coats by centrifugation at 250×*g* and room temperature for 10 min. Platelets were washed as previously described [9] and were resuspended in HEPES-buffered Tyrode buffer (pH 7.4) containing 2 mM Ca^{2+}. Platelet aggregation was measured turbidimetrically as previously described [10]. Aggregation values are given as the maximum change in light transmittance (100% was the difference between washed platelets and buffer). In some experiments, the effects of 4N1-1 on GPVI-depleted mouse platelets was studied as described previously [11]. Agglutination of erythrocytes and cultured cells was also measured turbidimetrically as described above. Erythrocyte agglutination was also evaluated macroscopically.

2.3. Measurement of $[Ca^{2+}]_i$

Platelet-rich plasma was loaded with fura-2/AM (5 μM) for 30 min at 37°C. Thereafter, platelets were isolated [12] and fluorescence was measured spectrofluorophotometrically (RF-5000, Shimadzu, Kyoto, Japan) at an excitation wavelength of 340 nm and an emission wavelength of 500 nm.

2.4. Western blotting

Platelets were lysed in 2× lysis buffer (1×: 62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% w/v sodium dodecyl sulfate (SDS), 0.1% w/v bromophenol blue). Proteins were resolved by SDS–polyacrylamide (10%) gel electrophoresis, blotted and probed with antibodies as previously described [13]. Densitometric analysis was performed using a calibrated scanner densitometer (GS-800, Bio-Rad, Munich, Germany).

2.5. Flow cytometry

Expression of CD62P was measured as previously described [12].

2.6. Cell culture

Human venous and arterial smooth muscle cells were isolated by explant technique [14]. The cell lines MEG-01, CMK and U937 were obtained from the German Collection of Microorganisms and Cell Cultures and cultured as described [13].

2.7. Statistics

Data are mean ± S.E.M. of *n* independent experiments. Statistical analysis was performed by one-way analysis of variance followed by Bonferroni multiple comparisons test using GraphPad InStat version 3.01 for Windows 95 (GraphPad Software, San Diego, CA, USA). *P* levels of < 0.05 were considered significant.

3. Results

3.1. Effects of 4N1-1 on platelet aggregation

4N1-1 concentration-dependently induced platelet aggregation (Fig. 1a). However, while 100 μM 4N1-1 consistently stimulated platelet aggregation in each sample studied, the effects of lower concentrations of 4N1-1 were variable [3]. Platelet agglutination, amounting to about 25% of the aggregation amplitude in native platelets, was also observed in paraformaldehyde-fixed platelets (Fig. 1b,c).

3.2. Effects of 4N1-1 on platelet signaling

4N1-1-induced aggregation was accompanied by increases in $[Ca^{2+}]_i$ with maximum values comparable to those induced by thrombin (0.1 U/ml). However, while the increases in $[Ca^{2+}]_i$ were very rapid upon stimulation with thrombin, peak increases in $[Ca^{2+}]_i$ in 4N1-1-stimulated platelets occurred after about 2 min (Fig. 2). 4N1-1 (100 μM) stimulated a time-dependent phosphorylation of Akt at Ser^{407} (Fig. 3a) and Thr^{308} (not shown) with a time course similar to that induced by convulxin (Fig. 3b). Akt phosphorylation induced by either stimulus was completely inhibited by the Src family tyrosine kinase inhibitor PP2 (10 μM) (Fig. 3c). Similar to convulxin (Fig. 3e), 4N1-1 (100 μM) also stimulated a time-dependent phosphorylation of p38 MAP kinase (Fig. 3d). Again, p38 MAP kinase phosphorylation induced by either stimulus was completely inhibited by PP2 (10 μM) (Fig. 3f). Interestingly, 4N1-1 did not stimulate ERK-1/2 phosphorylation (Fig. 3g), while thrombin or convulxin did (Fig. 3h).

3.3. Effects of 4N1-1 on aggregation of GPVI-deficient platelets

Together, the above described experiments demonstrated that 4N1-1 induces a different pattern of signal transduction pathways than the GPVI-specific agonist, convulxin. Therefore, the proposed involvement of GPVI signaling in 4N1-1-induced aggregation was tested directly in GPVI-deficient mouse platelets [11]. Strikingly, 4N1-1-induced aggregation was not altered in GPVI-deficient platelets clearly excluding an essential role of GPVI in this process (Fig. 4).

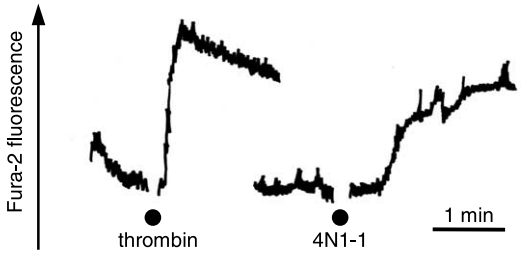

Fig. 2. Original tracings, demonstrating the effects of 4N1-1 (100 μM) as compared to thrombin (1 U/ml) on $[Ca^{2+}]_i$. Similar results were obtained in three independent experiments.

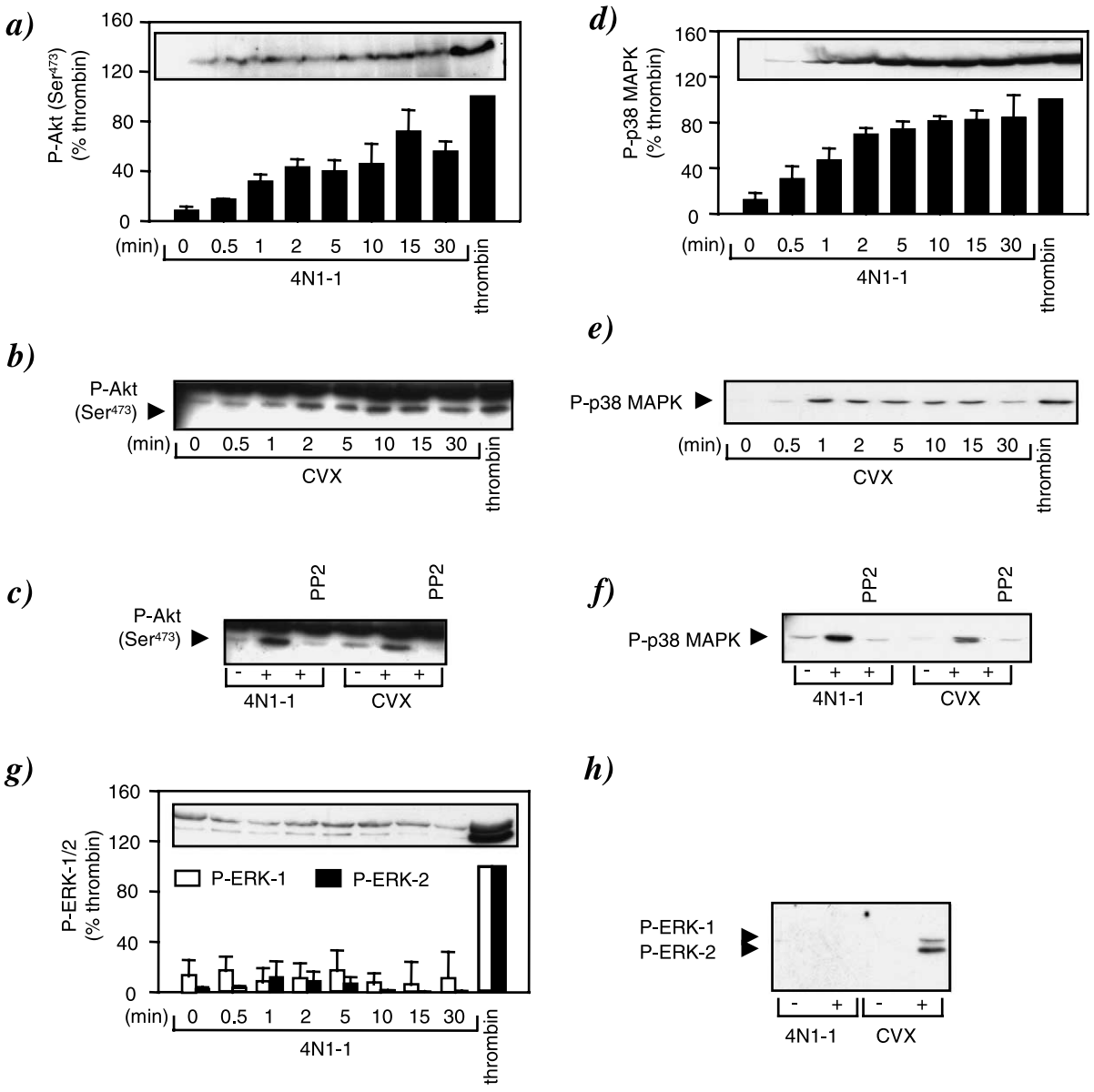

Fig. 3. Effects of 4N1-1 (100 μM) as compared to convulxin (CVX, 100 ng/ml) or thrombin (1 U/ml, 5 min) on phosphorylation of Akt (a,b), p38 MAP kinase (d,e), and ERK-1/2 (g,h). c,f: The effects of the Src family tyrosine inhibitor PP2 (10 μM). a,d,g: Representative Western blots and the densitometric analysis (means ± S.E.M.) of $n = 5$ experiments.

3.4. *4N1-1-induced aggregation is not altered by GPIIb/IIIa inhibitors*

In contrast to the previously published data [6], 4N1-1-induced platelet aggregation was not affected by high concentrations of different GPIIb/IIIa inhibitors (10 μM abciximab, a monoclonal antibody fragment; 3 μM eptifibatide, a cyclic peptide; 100 nM tirofiban, a peptide mimetic; 500 μM RGDS) (Fig. 5). In contrast, all inhibitors reduced 0.1 U/ml thrombin-induced aggregation by about 80% and completely blocked aggregation in response to 100 ng/ml convulxin (not shown). Together, these data demonstrate that 4N1-1-induced platelet aggregation is an entirely GPIIb/IIIa-independent process.

3.5. *Effects of platelet function inhibitors on 4N1-1-induced platelet aggregation*

The surprising finding that neither GPVI nor GPIIb/IIIa is

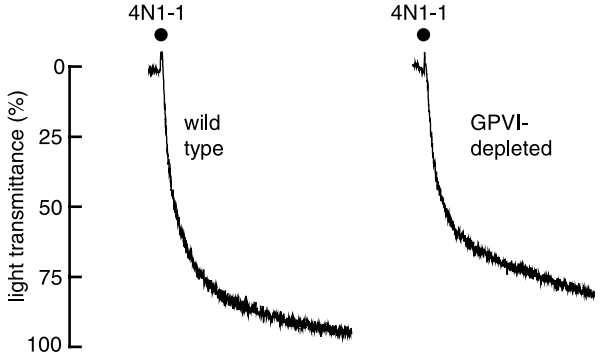

Fig. 4. Original tracings, demonstrating the aggregatory effects of 4N1-1 (250 μM) on platelets isolated from wild type mice and from GPVI-depleted mice. Similar results were obtained in three independent experiments.

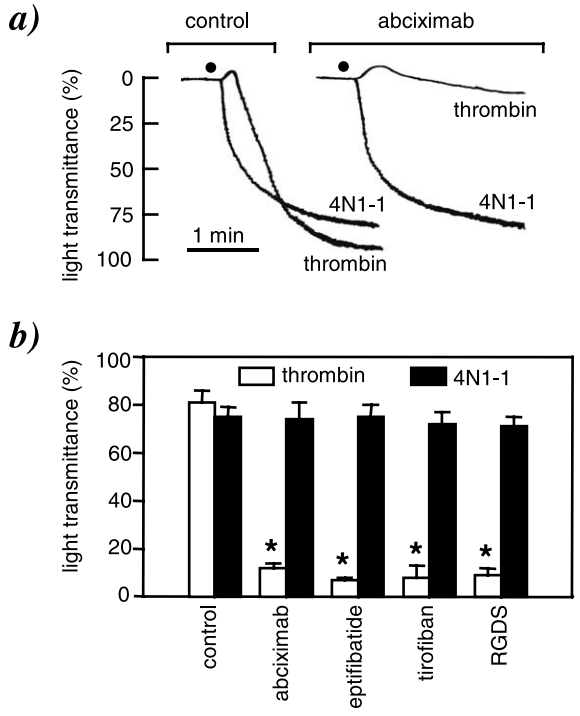

Fig. 5. Effects of various GPIIb/IIIa inhibitors on 100 μM 4N1-1- and 0.1 U/ml thrombin-induced platelet aggregation. a: Original tracings, demonstrating the effects of abciximab (10 μg/ml). b: Quantitative analysis of the effects of abciximab (10 μg/ml), eptifibatide (3 μM), tirofiban (100 nM), and RGDS (500 μM) (means ± S.E.M., $n = 5$, $^*P < 0.05$ versus control).

critically involved in 4N1-1-induced platelet aggregation prompted us to test the functional significance of signaling pathways activated by this agonist. For this purpose, platelets were pre-incubated with the cell-permeable Ca^{2+} chelator BAPTA/AM (100 μM), the phosphoinositide 3-kinase inhibitors wortmannin (300 nM) or LY-294002 (100 μM), or the p38 MAP kinase inhibitors SB202190 (10–100 μM) or SB203880 (10–100 μM) for 15 min and then stimulated with 4N1-1. Interestingly, none of these inhibitors altered the aggregation response (data not shown). Furthermore, 4N1-1-induced platelet aggregation was not affected by the cAMP-elevating agents iloprost (100 nM) or prostaglandin E_1 (300 nM) (not shown), or the cGMP-elevating agent sodium nitroprusside (SNP, 100 μM) (Fig. 6a,b). In contrast, platelet aggregation induced by thrombin (0.1 U/ml) (Fig. 6a,b) or convulxin (data not shown) was almost completely inhibited by iloprost or SNP. Interestingly, the activation of signal transduction pathways by 4N1-1, as exemplified by p38 MAP kinase phosphorylation, was almost completely inhibited by iloprost (Fig. 6c). Similarly, although platelet aggregation was not affected, iloprost markedly (about 50%) inhibited platelet α-granule secretion (CD62P expression, Fig. 6d). Thus, inhibition of some platelet function aspects (p38 MAP kinase phosphorylation, α-granule secretion) by iloprost did not result in any inhibition of 4N1-1-induced platelet aggregation.

3.6. 4N1-1-induced agglutination is not cell type-specific but is mediated by CD47

The results of the above described experiments clearly indicate that 4N1-1 might aggregate platelets independently of classical platelet receptors/signaling pathways. To test this hypothesis directly, the effects of 4N1-1 were tested on different megakaryocytic and non-megakaryocytic cells. As shown in Fig. 7a,b, 4N1-1 stimulated agglutination of two promegakaryocytic cell lines CMK and MEG-01 whereas no such effect was seen with convulxin (Fig. 7a,b) or thrombin (not shown). Interestingly, 4N1-1 induced p38 MAP kinase phosphoryla-

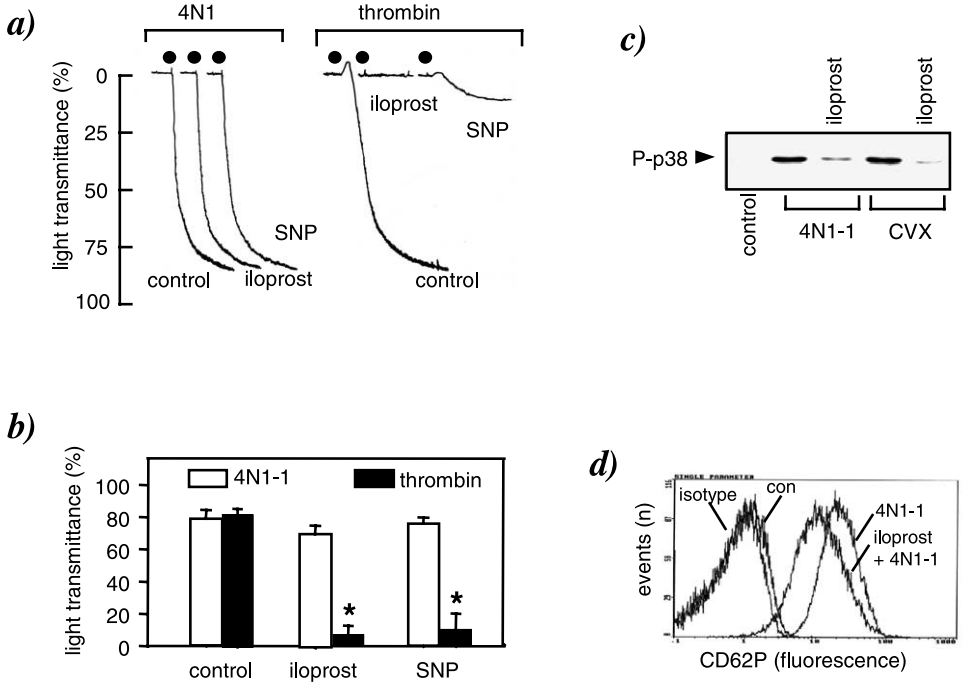

Fig. 6. Effects of iloprost (100 nM) and SNP (100 μM) on 100 μM 4N1-1-induced platelet aggregation (a,b), p38 MAP kinase phosphorylation (c), and α-granule secretion (CD62P expression) (d). In a,b, the effects of iloprost on 0.1 U/ml thrombin-induced platelet aggregation are shown for comparison (means ± S.E.M., $n = 5$, $^*P < 0.05$ vs. control).

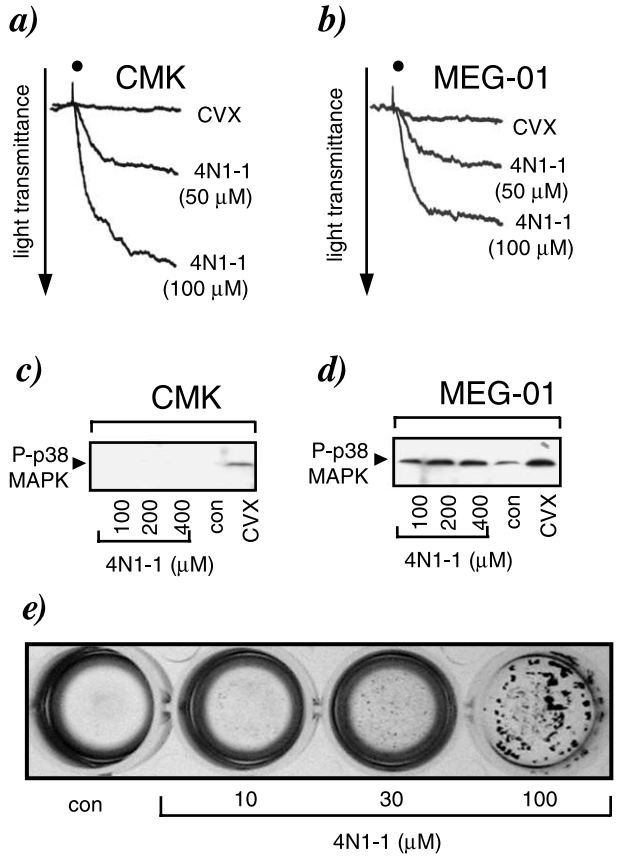

Fig. 7. Effects of 4N1-1 as compared to convulxin (CVX, 100 ng/ml) on agglutination of the megakaryocytic cell lines CMK (a) and MEG-01 (b), and erythrocytes (e). The effects of 4N1-1 and convulxin on p38 MAP kinase phosphorylation are shown in c,d. Similar results were obtained in two independent experiments.

tion in MEG-01 but not in CMK cells (Fig. 7c,d), further indicating an activation-independent agglutinatory effects of the peptide. This was confirmed by the observation that 4N1-1 also stimulated agglutination of non-megakaryocytic cells, including erythrocytes (Fig. 6e), arterial and venous smooth muscle cells, and the monocytic U937 cells (not shown). In contrast to the previously published data with CD47−/− mice [6], 4N1-1-induced aggregation of platelets (Fig. 8a) and agglutination of erythrocytes (Fig. 8b) was completely blocked by neutralizing anti-CD47 antibodies. However, these antibodies did not inhibit 4N1-1-induced platelet signaling (p38 MAP kinase phosphorylation) (Fig. 8c).

4. Discussion

The present study investigated the effects of 4N1-1 on several signaling pathways and the effects of different specific platelet inhibitors on 4N1-1-induced platelet aggregation.

4N1-1 has recently been proposed to signal through a pathway similar to that used by the collagen receptor GPVI [6] as the peptide induced tyrosine phosphorylation of the FcRγ chain, Syk, SLP-76 and phospholipase Cγ2. In addition, the phosphorylated FcRγ chain was shown to coprecipitate with GPVI. Although there is indirect evidence in non-platelet cells that the GPVI/FcRγ chain is not activated by 4N1-1 [6], suggesting that 4N1-1 does not directly activate GPVI in platelets, this issue has not yet been experimentally addressed. However, although 4N1-1 stimulated several platelet signaling events in a tyrosine kinase-dependent fashion, a different pattern of signal transduction pathways was observed upon stimulation with 4N1-1, as compared to the GPVI-specific agonist convulxin. For example, 4N1-1 did not stimulate ERK-1/2 phosphorylation, while convulxin did. In addition, using GPVI-deficient mouse platelets, the present study excludes a role of GPVI in 4N1-1-induced platelet aggregation.

In contrast to previous findings, where at least a partial inhibition of 4N1-1-induced aggregation by RGDS peptide was reported [6], no inhibitory effects of several GPIIb/IIIa inhibitors, including abciximab, tirofiban, eptifibatide, and RGDS, were observed in the present study. It may be difficult to explain these discrepancies at present but it is clear that the concentrations used in the current study were high enough to completely block surface GPIIb/IIIa receptors [15]. Accordingly, thrombin-induced platelet aggregation was inhibited by about 80% under these conditions. The residual platelet aggregation in the presence of GPIIb/IIIa inhibitors can be explained by the rapid fibrinogen binding kinetics to externalized GPIIb/IIIa receptors [12,16]. Similarly, convulxin-induced platelet aggregation was completely inhibited by GPIIb/IIIa inhibitors, thus indicating a further difference between 4N1-1- and convulxin/GPVI-mediated platelet aggregation.

The unexpected finding that 4N1-1-induced platelet aggregation occurred entirely independently of GPIIb/IIIa strongly suggested that classical platelet signaling pathways may not be critically involved in this process. This hypothesis was strongly confirmed by studies using specific inhibitors demonstrating that increases in $[Ca^{2+}]_i$, phosphorylation of Akt and p38 MAP kinase are not required for 4N1-1-induced platelet aggregation.

Furthermore, although 4N1-1-induced signaling (p38 MAP kinase phosphorylation), GPIIb/IIIa receptor activation (fibrinogen binding), and α-granule secretion (CD62P expression) were markedly inhibited by the prostacyclin mimetic iloprost, platelet aggregation was not affected at all. Similar findings were obtained with SNP. This was a striking and

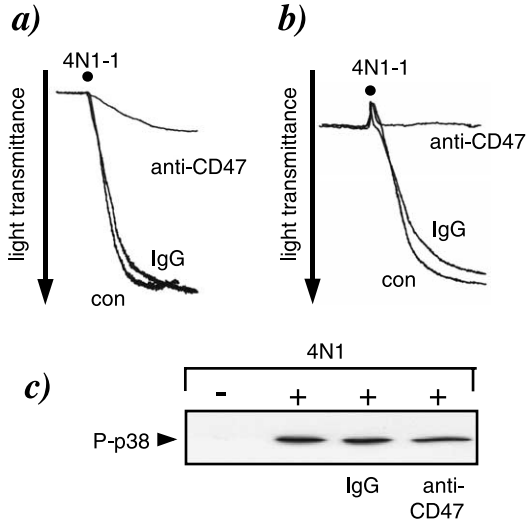

Fig. 8. Effects of neutralizing anti-CD47 antibodies (30 μg/ml) on platelet aggregation (a) and erythrocyte agglutination (b). The effects of neutralizing anti-CD47 antibodies on 4N1-1-induced p38 MAP kinase phosphorylation are shown in c. Similar results were obtained in two independent experiments.

unexpected finding, because elevation of either intracellular cAMP or cGMP concentrations is the most potent endogenous mechanism of platelet inhibition [17]. Although cAMP-insensitive signaling pathways in platelets, especially those mediated by GPVI, have been described [18–21], platelet aggregation induced by any known agonist, including GPVI activators, can be inhibited by elevation of cyclic nucleotides [17,22]. In agreement with this, platelet aggregation induced by convulxin or by thrombin, which is the strongest known platelet stimulus, was almost completely blocked by iloprost or by SNP.

Together, these observations demonstrate that 4N1-1 stimulates platelet aggregation in a GPVI- and GPIIb/IIIa-independent fashion. On the other hand, GPIbα has previously been shown to be not involved [6]. Furthermore, inhibition of α_2 integrins has no effect on 4N1-1-induced aggregation of mouse platelets platelet (Aktas and Nieswandt, unpublished observation). Finally, neither the inhibition of any signaling pathway induced by 4N1-1 nor the inhibition of platelet activation by elevation of cyclic nucleotides had an effect on 4N1-1-induced aggregation, strongly suggesting that 4N1-1 might aggregate platelets independently of classical platelet receptors/signaling pathways. This was confirmed by the finding that 4N1-1 stimulated agglutination of two promegakaryocytic cell lines (CMK and MEG-01) and non-megakaryocytic cells, including erythrocytes, arterial and venous smooth muscle cells and the monocytic cell line U937. Interestingly, 4N1-1 stimulated p38 MAP kinase phosphorylation in MEG-01 but not in CMK cells, further indicating that stimulation of intracellular signaling events, such as p38 MAP kinase phosphorylation, might not be required for the effects on cell agglutination. Furthermore, experiments with neutralizing antibodies strongly suggest that CD47 might mediate the agglutinating effects of 4N1-1. This is in contrast to data obtained with CD4−/− mice [6]. On the other hand, 4N1-1-induced platelet aggregation on immobilized collagen was completely abolished in CD47−/− mice [5] and a direct binding of 4N1-1 to CD47 could be demonstrated [3]. In addition, in the study by Tulasne et al. [6], $F(ab')_2$ fragment of anti-CD47 antibodies did not inhibit 4N1-1-induced platelet aggregation. However, in previous studies this antibody was shown to block 4N1-1-induced spreading of platelets on immobilized fibrinogen [4]. One possible explanation for these divergent findings may be the fact, that in our study, as well as in the study by Chung et al. [4], the whole antibody (also B6H12) was used.

Interestingly, neutralizing anti-CD47 antibodies did not inhibit 4N1-1-induced p38 MAP kinase phosphorylation in platelets, indicating that 4N1-1 might also bind to receptors other than CD47.

It is concluded that 4N1-1 stimulates aggregation of platelets by an activation-independent mechanism which does not involve GPVI signaling. 4N1-1-induced aggregation is not dependent on GPIIb/IIIa receptors and also occurs in non-megakaryocytic cells. CD47 may mediate cell agglutination induced by 4N1-1.

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