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Adrenoceptor α_{2A} signalling countervails the taming effects of synchronous cyclic nucleotide-elevation on thrombin-induced human platelet activation and aggregation

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ABSTRACT

The healthy vascular endothelium constantly releases autacoids which cause an increase of intracellular cyclic nucleotides to tame platelets from inappropriate activation. Elevating cGMP and cAMP, in line with previous reports, cooperated in the inhibition of isolated human platelet intracellular calcium-mobilization, dense granules secretion, and aggregation provoked by thrombin. Further, platelet alpha granules secretion and, most relevant, integrin $\alpha_{IIa}\beta_3$ activation in response to thrombin are shown to be prominently affected by the combined elevation of cGMP and cAMP. Since stress-related sympathetic nervous activity is associated with an increase in thrombotic events, we investigated the impact of epinephrine in this setting. We found that the assessed signalling events and functional consequences were to various extents restored by epinephrine, resulting in full and sustained aggregation of isolated platelets. The restoring effects of epinephrine were abolished by either interfering with intracellular calcium-elevation or with PI3-K signalling. Finally, we show that in our experimental setting epinephrine likewise reconstitutes platelet aggregation in heparinized whole blood, which may indicate that this mechanism could also apply in vivo.

1. Introduction

Human blood platelets, small non-nucleated cells which are shed off by megakaryocytes, patrol the blood vessels as sentinels of vascular integrity. In the course of primary haemostasis platelets instantly adhere to the denuded sub-endothelium at the site of vascular injury, become activated and release autocrine mediators, enable the fibrinogen-receptor integrin $\alpha_{IIb}\beta_3$, and aggregate to build a fibrinogen-bridged plug that seals the breach within seconds to minutes. Due to their vigorous reactivity, platelets require perpetual but likewise balanced repression; to allow for physiological haemostasis as well as to prevent from pathological thrombus formation, the latter which in the worst case may occlude vessels resulting in ischemic events such as myocardial infarction or stroke [1]. The healthy vascular endothelium, in which close vicinity platelets travel the bloodstream, continuously release nitric oxide (NO) and prostacyclin, the latter which is also designated prostaglandin I_2 (PGI_2). Whereas NO is membrane-permeable and directly activates intracellular guanylyl cyclase which gives rise to cyclic guanosine monophosphate (cGMP), PGI_2 activates cell-surface receptors, the latter that are abbreviated as IP receptors, which couple to the family of G protein-coupled receptors (GPCRs) that induce Gαs-signalling leading to the stimulation of adenylyl cyclase and, in turn, to an increase in cyclic adenosine monophosphate (cAMP) [2]. Human platelet A_{2A} and A_{2B} receptors for adenosine, the latter which is as well released by the endothelium or derive from adenine nucleotide breakdown, likewise evoke an elevation of cAMP via Gαs signalling [3]. The homoeostasis of platelet cyclic nucleotides is achieved via a negative feedback loop inducing phosphodiesterases (PDEs) which the breakdown cGMP and cAMP.

However, the elevation of cyclic nucleotides causes activation of the cGMP- and cAMP-dependent protein kinases G (PKG) and A (PKA), respectively, leading to the phosphorylation of a broad spectrum of substrate proteins which in turn block almost all events involved in platelet activation and aggregation, such as cytoskeletal re-arrangements, G-protein activation, intracellular calcium (Ca^{2+}) mobilization, release of granules, and integrin $\alpha_{IIa}\beta_3$ activation. Interestingly, both PKG and PKA not only share several downstream targets but also some phosphorylation sites within the respective substrates, such as in the

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small G protein Rap1B and Rap1BGAp2, or the actin-binding proteins LASP (LIM and SH3 domain protein), HSP27 (heat shock protein 27), and VASP (vasodilator-stimulated phosphoprotein) [2].

The negative regulation of platelet activation by cyclic nucleotides applies to virtually every physiological platelet agonist and respective signalling pathway employed, including ligands of GPCRs such as thrombin, thromboxane A_2 (TXA_2), and adenosine diphosphate (ADP); as well as collagen, von Willebrand factor (vWF), and fibrinogen [2,4].

Thrombin, also known as activated Factor II (FIIa), is massively produced during thrombus-formation ('thrombin-burst') at the site of vascular injury and is the main effector protease of the coagulation cascade provoking the activation of human platelets mainly via protease-activated receptors (PARs) 1 and 4 [5]. Both PAR1 and PAR4 induce Gα12/13 and Gαq signalling [6]. Whereas the initial reorganization of the platelet cytoskeleton ('shape change') is mediated via Gα12/13-induced calcium/calmodulin and Rho/Rho-kinase signalling pathways, Gαq signalling is required for platelet granule secretion which is accomplished via phospholipase C (PLC) β signalling and subsequent Ca^{2+}- and diacylglycerol (DAG)-provoked activation of protein kinase C (PKC), the latter which is strongly implicated in the secretion of platelet granules, such as dense granules which contain adenosine triphosphate (ATP) and ADP.

The P2X1 ATP receptor is a ligand-gated ion channel, inducing a rapid calcium influx associated with transient shape change of human platelets [7]. For ADP the Gαq-coupled P2Y1 receptors and Gαi2-coupled P2Y12 receptors are expressed on platelets. The β/γ-subunit of Gαi directly activates phosphatidylinositol 3-kinase (PI3-K) which amplifies granule secretion as well as activation of integrin $\alpha_{IIa}\beta_3$ (by a mechanism termed 'inside-out' signalling) whereas the α-subunit downregulates adenylyl cyclase (AC) and therefore cAMP levels, both which are pivotal for full and sustained platelet activation and aggregation [8].

All physiological agonists known so far critically rely on the release of ADP from dense granules and subsequent signalling via P2Y12 receptors regardless of their initial signalling cascade engaged [9]. Hence, P2Y12 receptor antagonists, such as clopidogrel or ticagrelor are highly effective in preventing platelet activation and aggregation and are therefore in clinical use for several years.

Considering the significance of the signalling events induced by inhibitory Gα proteins, it is most noteworthy that platelets also express a remarkable number of α_{2A} adrenoceptors, which couple to inhibitory Gαz proteins [10]. Compared with 425 ± 50 copies of P2Y12 receptors [11], approximately 300 copies of α_{2A} adrenoceptors per platelet have been reported [12,13]. Epinephrine is considered to be a weak agonist that does not provoke activation and aggregation of isolated human platelets on its own but to amplify platelet responses induced by a primary stimulus such as thrombin. Along this line, on a molecular level, it has been shown that preventing platelet aggregation induced by ADP or thrombin by P2Y12 receptor antagonists can be bypassed by epinephrine via α_{2A} adrenoceptor signalling [14,15].

Since more than half a century it has been reported in numerous studies that under acute myocardial infarction sympathetic nervous activity is augmented resulting in increased plasma levels of catecholamines [16–19]. Although it is well established that activation of the sympathetic nervous system correlates with platelet hyperreactivity, the intracellular molecular mechanisms involved in vivo are less well established.

By using isolated human platelets we investigated how the catecholamine epinephrine may modulate thrombin-induced platelet reactivity and responses in vitro under conditions mimicking those which repress platelet functional responses in vivo, i.e. in the presence of cyclic nucleotide-elevating compounds. We found that epinephrine easily counteracts the platelet-taming effects of concurrent cGMP and cAMP elevation by provoking signalling events restoring functional consequences such as granules secretion, integrin $\alpha_{IIa}\beta_3$ activation, and aggregation. Epinephrine likewise reconstituted platelet aggregation in heparinized whole blood.

Taken together, it appears that synchronous PKG/PKA-signalling, whereas effectively blocking platelet activation in response to a primary agonist such as thrombin, has no impact on the signalling cascades triggered by α_{2A} adrenoceptors.

2. Materials and methods

2.1. Chemicals and antibodies

Apyrase (Grade III, from potatoes), fura-2/AM (fura 2 acetoxymethylester), bovine thrombin (T4648), epinephrine, SNAP (S-Nitroso-*N*-acetyl-DL-penicillamine), adenosine, PGI_2 (prostaglandin I_2), BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester), fibrinogen, yohimbine, and DMSO (dimethyl sulfoxide) were from Sigma-Aldrich (Stockholm, Sweden). LY294002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride), wortmannin, MRS2179 (2′-Deoxy-N^6-methyladenosine 3′,5′-bisphosphate tetrasodium salt) and NF449 (4,4′,4″,4‴-[Carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino))]tetrakis-1,3-benzenedisulfonic acid, octasodium salt) were from Tocris (Bristol, UK). Heparin (LEO Pharma, Malmö, Sweden), Cangrelor (Kengreal®, Chiesi USA, Inc.) and tirofiban (Aggrastat®, Correvio (UK) Limited) were purchased from the local University'spharmacy. The PAR1 and PAR4-activating peptides SFLLRN and AYPGKF-NH_2, respectively, were custom-synthesized by JPT Peptide Technologies (Berlin, Germany). Convulxin was purchased from Alexis/Enzo Life Sciences, Inc. (Farmingdale, NY, USA). The phospho-Akt Thr^{308} and Ser^{473} antibodies (#4056 and #4051), the phospho-VASP Ser^{157} and Ser^{239} antibodies (#3111 and #3114), as well as the secondary goat anti-rabbit and horse anti-mouse HRP-linked IgGs (#7074 and #7076) were from Cell Signaling Technology, Inc. (Danvers, MA, USA), while the pan-Akt antibody (ab8805) was bought from Abcam (Cambridge, UK). Antibodies and isotype controls applied for flow cytometry analysis were from BD Biosciences (Franklin Lakes, NJ, USA), except for anti CD41a, which was from Beckman Coulter Inc. (Brea, CA, USA). All other reagents were of analytical grade.

2.2. Isolation of human platelets

With approval from the regional ethical board Örebro-Uppsala county (Dnr 2015/543) heparinized blood was obtained from healthy volunteers, who denied to have taken any medication two weeks prior to donation, was drawn into syringes containing 10 IU heparin per ml blood, transferred into tubes containing acid-citrate-dextrose ((ACD) 71 mM citric acid, 85 mM sodium citrate, 111 mM glucose) in a volumetric proportion of 5:1, and centrifuged at 220*g* for 20 min. The platelet-rich plasma thus obtained was supplemented with 1 U/ml apyrase, and platelets were collected by centrifugation at 480*g* for 25 min. Platelet pellets were carefully washed three times with KRG (Krebs-Ringer glucose) buffer (120 mM NaCl, 4.9 mM KCl, 1.2 mM$MgSO_4$, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4 and 10 mM glucose, pH 7.3), and finally resuspended in KRG containing 1 U/ml apyrase to prevent any desensitizing effects of during the resuspension process possibly released ADP and ATP on P2Y1 and P2Y12 receptors, and especially P2X1 receptors, respectively. [7]. The platelet count was determined using an automatic blood cell counter (ABX Diagnostics Micros 60) and was adjusted to the concentration required for the respective assay. Platelet suspensions were supplemented with 1 mM $CaCl_2$ 30 min prior to experimentation. All isolation steps were carried out at room temperature.

2.3. Aggregation and ATP release using isolated platelets

Measurements were using Chrono-Log lumi-aggregometer (Model 700) with using a final volume of 0.3 ml suspension of isolated platelets adjusted to 2.5×10^8 platelets/ml. Isolated platelets were preincubated

and stimulated at 37 °C with stirring at 900 rev./min. Aggregation is expressed as percentage light transmission compared with KRG alone (=100%). Extracellular ATP was assessed by applying a luciferin/luciferase bioluminescence assay and calculated using an exogenously added ATP standard according to the manufacturer's protocol (Chrono-Log, Havertown, PA, USA).

2.4. Aggregation in whole blood

Heparinized blood was diluted 1:1 with KRG buffer containing 1 mM $CaCl_2$ without apyrase and platelet aggregation was measured at 37 °C with stirring at 900 rev./min in a volume of 1.8 ml by impedance, which increases between two platinum electrodes when platelets aggregate on their surface.

2.5. Increase in cytosolic Ca^{2+}

Platelets were loaded with fura 2 by incubating platelet-rich plasma with 4 μM fura 2/AM for 45 min at room temperature in the dark and subsequently isolated as described in section 2.2. The platelet count was adjusted to 0.125×10^8/ml and 1 mM $CaCl_2$ was supplemented prior to experimentation. Platelets were preincubated and stimulated as indicated at 37 °C with gentle stirring and fluorescence was recorded using a Hitachi F-7000 spectrofluorimeter at 510 nm with simultaneous excitation at 340 nm and 380 nm. Cytosolic calcium $[Ca^{2+}]_i$ is expressed as a fluorescence ratio (340/380 nm).

2.6. Flow cytometry

The level of platelet activation was analysed using a four colour panel containing the platelet marker (anti-CD41a-ECD) and three activation markers (anti-CD62p-PE, anti-CD63-PE-Cy7 and PAC-1-FITC). Corresponding isotype controls were applied. Stimulation of isolated platelets (0.125×10^8/ml) was carried out at 37 °C in a total volume of 300 μl in 2 ml round-bottom tubes in a thermoshaker rotating at 300 rpm. Platelets were pretreated with vehicle (0.05% DMSO), or both 10 μM SNAP and 10 μM adenosine for 2 min, followed by 0.3 U/ml thrombin, or with thrombin for 3 min followed by 20 μM epinephrine. For one set of experiments, platelets were preincubated with vehicle, or LY294002, or BAPTA/AM prior to the above mentioned experimental procedure. Reactions were stopped after 3 and/or 6 min by transferring 9 μl platelet suspension into 23,5 μl KRG containing 0.2% formaldehyde. Subsequently, antibodies were added and platelets were stained for 10 min before diluting the sample for acquisition with another 200 μl KRG/0.2% formaldehyde. Data acquisition of 10,000 events was performed using a Gallios Flow Cytometer and data analysis was performed using Kaluza software v1.5 (both Beckman Coulter, Inc., Stockholm, Sweden). The platelets were identified based on the expression of CD41a, and results are presented as percentage positive platelets (CD41a+).

2.7. Immuno(Western) blotting

Stimulation of isolated platelets (2.5×10^8 platelets/ml) was carried out at 37 °C in a total volume of 200 μl in 2 ml round-bottom tubes in a thermoshaker rotating at 900 rpm; pre-incubations at 500 rpm. Reactions were stopped by the addition of 50 μl 5× SDS sample buffer, and proteins were denaturated at 95 °C for 5 min. Proteins were separated on 4–12% NuPAGE® Novex Bis-Tris gels with MOPS running buffer (Invitrogen, Stockholm, Sweden). To determine apparent molecular protein masses MagicMark™ XP Western Protein Standard (Invitrogen) was used. Proteins were blotted onto Immun-Blot™ PVDF membranes (0.2 μm) (BioRad, Hercules, CA, USA). For further steps TBS-T (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% (*w/v*) Tween-20) was used. For assessing Akt phosphorylation membranes were incubated with either anti-phospho-Akt Thr308 or Ser473 antibodies (both at a 1:1000 dilution) followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgGs or horse anti-mouse IgGs, respectively (both diluted 1:2000). For determining VASP phosphorylation membranes were incubated with either anti-phospho VASP Ser157 or Ser239 antibodies (diluted 1:1000) followed by HRP-conjugated goat anti-rabbit IgGs (1:2000). For reprobing membranes were stripped and incubated with the pan-Akt antibody (1:1000) followed by HRP-conjugated goat anti-rabbit IgGs (1:2000). Protein bands were visualized by the use of Immobilon™ Western Chemiluniescent HRP Substrate solution from Millipore (Billerica, MA, USA), and chemiluminescence was recorded by a Fuji LAS 1000 system (Fuji Photo Film, Tokyo, Japan).

2.8. Statistical analysis

Data analysis was performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Results are presented as means ± S.E.M. and statistical significance was calculated as indicated.

3. Results

3.1. Signalling induced by elevating cyclic nucleotides cooperate in the inhibition of thrombin-provoked dense granules secretion and aggregation of isolated platelets

The elevation of either cGMP by 10 μM SNAP or of cAMP by 10 μM adenosine, as shown in Fig. 1A and B, abrogate dense granules secretion (in terms of ATP release) as well as aggregation (in terms of light transmission) of platelets challenged with an intermediate thrombin-concentration of 0.1 U/ml. When platelets were stimulated with 0.3 U/ml the secretion of dense granules was to reduced by SNAP but not or nonsignificantly by adenosine, whereas platelet aggregation was not affected. In contrast, as demonstrated in Fig. 1C, the synchronous elevation of both cGMP and cAMP by SNAP and adenosine, respectively, cause a complete lack of ATP release and aggregation of platelets stimulated with 0.3 U/ml thrombin.

Platelet dense granules secretion in terms of ATP release and aggregation in terms of light transmission were determined in parallel as described in “Material and Methods”. Isolated human platelets were incubated vehicle (0.05% DMSO) or 10 μM SNAP (A) or 10 μM adenosine (Ado) (B), or both (C) for 2 min prior to the addition of 0.1 or 0.3 U/ml thrombin as indicated. The bar graphs show maximal ATP release (in nmole) and % light transmission assessed within and after 10 min of stimulation, respectively. Data of 3–4 individual experiments is presented as mean ± S.E.M. and statistical analysis were performed by two-tailed paired Student's *t*-tests (A, B) or one-way ANOVA followed by Bonferroni's multiple comparison test (C); $^*P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$, ns = not significant.

3.2. The inhibition of thrombin-induced granules secretion, integrin $\alpha_{IIb}\beta_3$ activation, and aggregation by elevated cGMP and cAMP is abrogated by epinephrine

Due to the complete absence of dense granules secretion as determined by ATP release as shown above in Fig. 1C, we investigated if the concurrent lack of released ADP and therefore P2Y12-mediated Gαi signalling may be the ultimate cause of the absence of platelet responses by substituting ADP with epinephrine. As illustrated by original traces in Fig. 2A, and as a summary in the bar graph in 2B, in platelets preincubated with both SNAP and adenosine (each at 10 μM) and challenged with 0.3 U/ml thrombin a subsequent addition of 20 μM epinephrine provokes a partial reconstitution of ATP secretion as well as a full aggregation response. Interestingly, when we changed the sequence of stimulation by adding epinephrine prior to thrombin, we likewise observed reconstituting effects on ATP secretion and aggregation upon thrombin-stimulation, indicating that the effect of

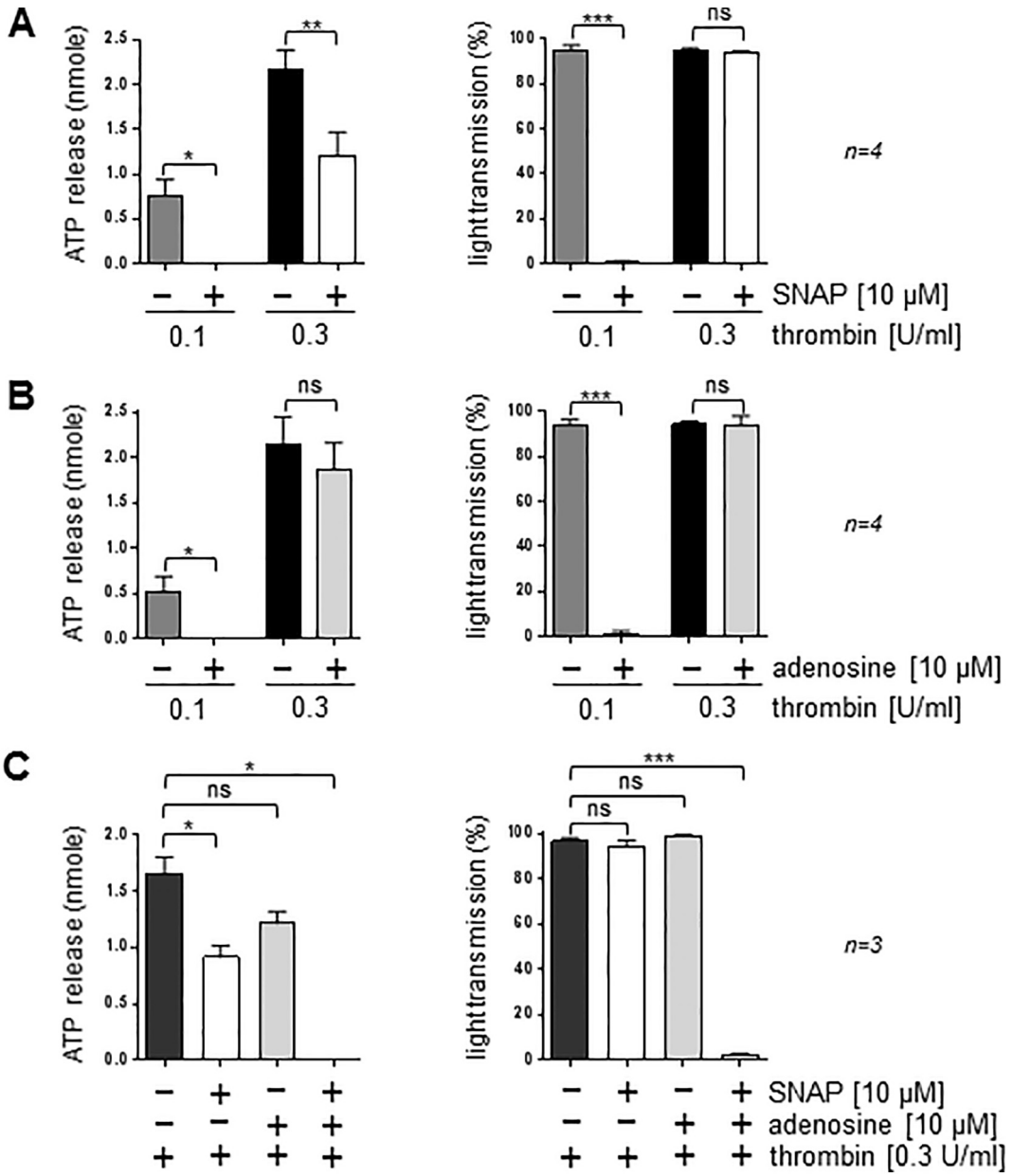

Fig. 1. The effects of elevating cGMP and cAMP cooperate in the inhibition of thrombin-induced dense granules secretion and aggregation of isolated platelets.

epinephrine has both priming as well as restoring properties. Further, as shown in Fig. 2C, the reconstituting effects of epinephrine is fully blocked by yohimbine (10 µM), demonstrating that the effect of epinephrine is mediated via α_{2A} adrenoceptors.

Platelet dense granules secretion in terms of ATP release and aggregation in terms of light transmission were determined in parallel as described in "Material and Methods". Isolated human platelets were preincubated with vehicle (0.05% DMSO) or both 10 µM SNAP and 10 µM adenosine (Ado) for 2 min prior to the addition of 0.3 U/ml thrombin (Thr) alone, or the addition of 0.3 U/ml thrombin followed by 20 µM epinephrine (Epi) after 3 min as indicated, or the addition of 20 µM epinephrine followed by 0.3 U/ml thrombin after 3 min as indicated. (A) shows original traces of platelet ATP release and light transmission which are representative of 4 independent experiments, which are summarized in the bar graphs shown in (B). In (C) bar graphs of ATP release and maximal light transmission from experiments in which platelets were additionally pretreated with 50 µM yohimbine 2 min prior to the addition of SNAP and adenosine was followed by thrombin and epinephrine are shown. In (D) flow cytometric analyses of platelet surface changes are presented. Surface expression of the secretion biomarkers LAMP-3 (Lysosome-Associated Membrane Protein 3, CD63), P-selectin (CD62P), as well as PAC-1 binding to the high-affinity conformation of integrin $\alpha_{IIa}\beta_3$, were analysed in isolated platelets as described in "Material and Methods". Isolated human platelets were preincubated with vehicle (0.05% DMSO), or both 10 µM SNAP and 10 µM adenosine for 2 min prior to the addition of 0.3 U/ml thrombin alone (solid lines), or the addition of 0.3 U/ml thrombin followed after 3 min by 20 µM epinephrine for another 3 min (dotted lines). Samples were analysed at time point 0 min (prior to stimulation), and 3 and 6 min after stimulation. Platelets were identified as integrin subunit α_{IIa} positive (CD41a+) cells and surface marker-positive cells are quantified as % of CD41a + cells. Reductions or increases in platelet surface changes are indicated by arrows. All graphs show data of 4 individual experiments and is presented as mean ± S.E.M. and statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison test; $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$, ns = not significant.

In addition, we investigated further aspects of platelet activation by

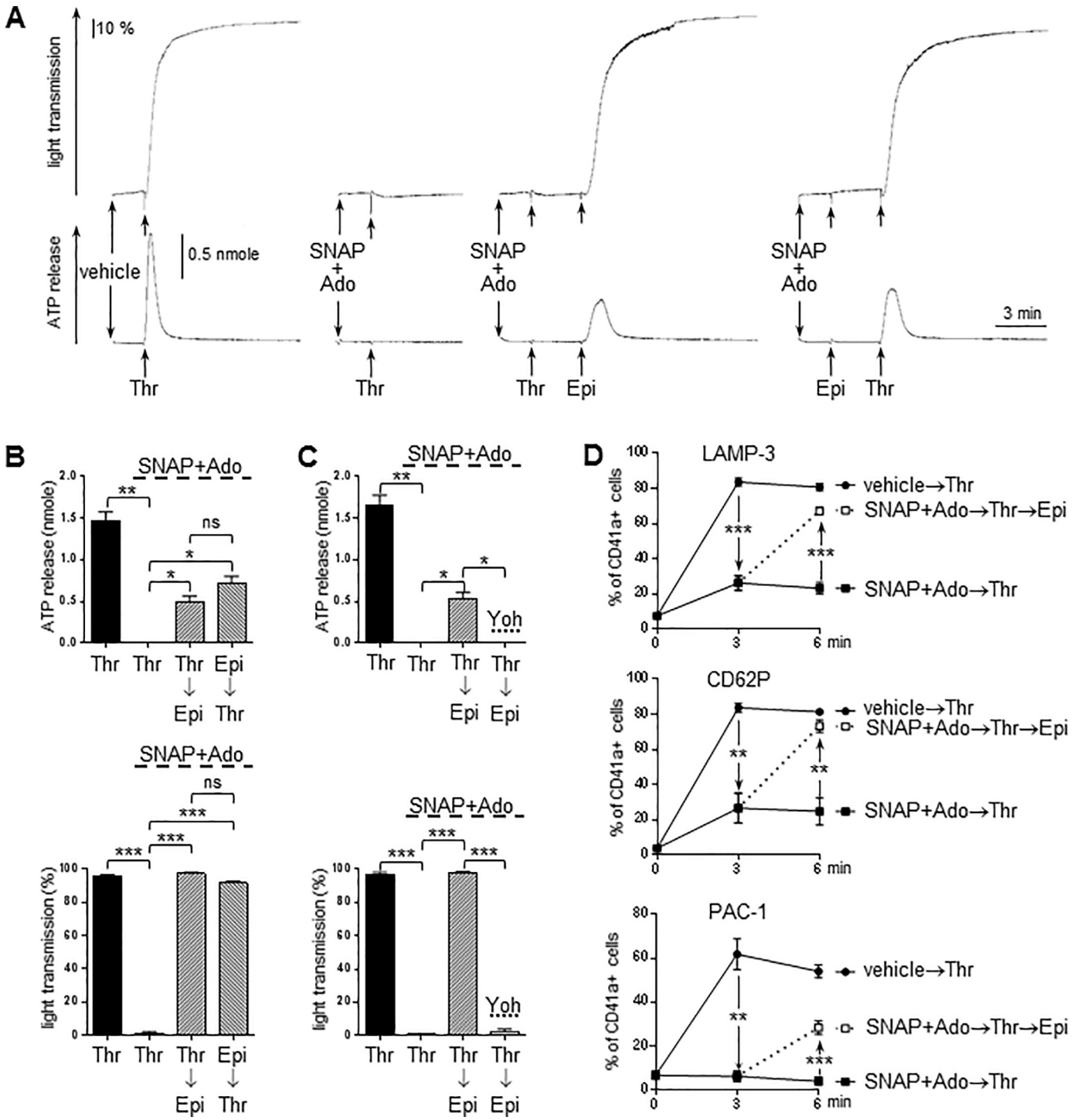

Fig. 2. The inhibition of thrombin-induced granules secretion, integrin $\alpha_{IIb}\beta_3$ activation, and aggregation by elevated cGMP and cAMP is abrogated by epinephrine.

assessing changes in the surface-expression of specific and commonly used platelet activation markers (Fig. 2D), such as LAMP-3 (Lysosome-Associated Membrane Protein 3) which reflects the secretion of lysosomes as well as dense granules, CD62P (P-selection) representing alpha-granules secretion [20], as well as PAC-1 which mirrors the conformational change and hence the activation of the fibrinogen-receptor integrin $\alpha_{IIb}\beta_3$ [21]. It may be worth mentioning that we performed these studies under the very same conditions we applied for the measurement of intracellular calcium-mobilization; i.e. using isolated platelets at a concentration of 0.125×10^8 platelets/ml under stirring conditions at 37 °C. For LAMP-3 we observed a significant increase in surface expression from 7.64 ± 0.67% on control platelets to 83.60 ± 2.27% in thrombin-stimulated platelets after 3 min of stimulation ($n = 4$, $P \leq .001$), which slightly dropped to 80.75 ± 2.03% after 6 min. Preincubation of platelets with SNAP and adenosine 2 min prior to thrombin-stimulation resulted in a significant inhibition of LAMP-3 expression after 3 min of incubation (down to 26.25 ± 4.09%) as well as after 6 min (23.25 ± 3.29%, $P \leq .01$, significance not indicated in the graph). However, in platelets pretreated with SNAP and adenosine and subsequently stimulated with 0.3 U/ml thrombin for 3 min followed by 20 μM epinephrine for another 3 min (total 6 min), a significant increase and reconstitution of LAMP-3 expression up to

67.10 ± 2.12% could be observed. Regarding CD62P (P-selectin) expression we obtained similar results. Thrombin alone evokes CD62P surface expression from 3.87 ± 0.58% on resting platelets up to 83.70 ± 2.45% after 3 min of stimulation ($n = 4$, $P \leq .001$) and 81.37 ± 1.96% after 6 min. Pretreatment with SNAP and adenosine in combination resulted in significant inhibition of CD62P expression on thrombin-stimulated platelets down to 26.68 ± 8.57% after 3 min, and down to 24.91 ± 7.76% ($P \leq .001$, significance not indicated in the graph) after 6 min of incubation, respectively.

Again, in platelets pretreated with SNAP and adenosine which were subsequently challenged with 0.3 U/ml thrombin followed by epinephrine (total 6 min), a significant increase and prominent reconstitution of CD62P expression up to 73.32 ± 3.66% could be observed. Comparable results were likewise assessed for integrin $\alpha_{IIb}\beta_3$ activation; PAC-1 binding increased from 6.77 ± 1.80% on resting platelets to 61.81 ± 6.95% after 3 min of thrombin-stimulation and slightly declined to 54.02 ± 3.03% after 6 min. After preincubation with the combination of SNAP and adenosine, PAC-1 binding was completely missing when platelets were stimulated with thrombin. This full inhibition, however, was significantly counteracted and PAC-1 binding re-established up to 28.39 ± 3.03% when epinephrine was additionally introduced. Epinephrine alone at 20 μM did not induce any changes in the surface composition of control platelets (data not shown). Finally, we observed differences in terms of dense granules secretion assessed by the luciferin/luciferase assay compared to that obtained by LAMP-3 expression (see also Fig. 4). This variation may be explained by the method of detection and their dynamics, which is continuous in the former assay where released ADP becomes steadily scavenged by the apyrase present and, in the latter, is an end-point measurement of the surface expression of LAMP-3 that is not affected by apyrase. It may strike one odd that although the introduction of epinephrine restored PAC-1 and therefore integrin $\alpha_{IIb}\beta_3$ to only about half of the maximal extent (Fig. 2D), platelet aggregation appear to be not even in part diminished (Fig. 2B.). This, however, may also be attributed to the various applied methods. Platelet aggregation measurements were performed in suspensions containing 2.5 × 108 platelets/ml with stirring at 900 rpm to favour fibrinogen-binding and aggregation, whereas flow cytometry experiments were carried out at 300 rpm and a concentration of 0.125 × 108 platelets/ml in order to promote PAC-1 binding and to prevent fibrinogen-binding. However, even when PAC-1 binding in the latter assay was found to be half-maximal, the number of activated integrin $\alpha_{IIb}\beta_3$ appeared to be high enough for sufficient fibrinogen-binding and full platelet aggregation in the former assay.

3.3. The suppression of thrombin-induced mobilization of intracellular calcium $[Ca^{2+}]_i$ by elevated cyclic nucleotides is counteracted by epinephrine

We further assessed cytosolic Ca^{2+}-mobilization, recognized as probably the most sensitive marker for platelet activation. Original traces obtained under our experimental conditions are shown in Fig. 3A and the results of three individual experiments are summarized in Fig. 3B.

Rises of $[Ca^{2+}]_i$ in isolated human platelets were determined as described in “Materials and Methods”. In (A) original traces of platelet $[Ca^{2+}]_i$ mobilization are presented. Isolated platelets were pretreated for 2 min with vehicle (0.05% DMSO), 10 μM SNAP, 10 μM adenosine (Ado), or SNAP and adenosine in combination, and then challenged with 0.3 U/ml Thrombin (Thr) followed after 3 min by 20 μM epinephrine (Epi) for 1 additional minute. In (B) the left graph shows the peak rises in intracellular $[Ca^{2+}]_i$ induced by thrombin and epinephrine, respectively, of 4 individual experiments and only the mean values are shown for reasons of lucidity. The right bar graph shows the data summarized as area under the curve (AUC) for the effects of thrombin alone within 3 min of stimulation (left part, AUC (1–3 min)) and the effects of epinephrine in the following minute (right part, AUC 3–4 min). Statistical analysis was performed by two-tailed paired Student's *t*-tests and indicated significances are compared to thrombin alone (AUC 1–3 min) or thrombin followed by epinephrine alone (AUC 3–4 min); $^*P \leq .05$, $^{***}P \leq .001$, ns = not significant.

Elevating cAMP with adenosine or cGMP with SNAP slightly affect the peak rise as well as the extent of a transient Ca^{2+}-mobilization provoked by thrombin, whereas when cAMP and cGMP are increased simultaneously we observe a synergistic inhibitory effect. Under every condition, however, a second rise in cytosolic Ca^{2+}-mobilization is caused by epinephrine, which appears to be most prominent when both cyclic nucleotides were previously elevated in parallel. As shown in the bar graph in Fig. 3B, analyzing the traces of Ca^{2+}-mobilization in more detail by calculating the area under the curve revealed that the inhibitory effect of either SNAP or adenosine on the Ca^{2+}-mobilization evoked by thrombin (left part of the bar graph; AUC 0 → 3 min) is not significant, whereas when SNAP and adenosine were combined the blockage is significantly pronounced. Regarding the second rise in Ca^{2+}-mobilization in response to epinephrine (right part of the bar graph; AUC 3 → 4 min), we observe no significant difference between non-preincubated platelets and those preincubated with SNAP or adenosine, whereas the rise in calcium is significantly higher in platelets when cGMP and cAMP were synchronously elevated. Finally, and in line with the results shown in Fig. 2B, the effects of epinephrine on Ca^{2+}-mobilization were blocked by yohimbine (10 μM), again giving proof that the effect of epinephrine is mediated via α_{2A} adrenoceptors (Fig. 3A, inset).

3.4. The elevation of intracellular calcium $[Ca^{2+}]_i$ is pivotal for the reconstituting effects of epinephrine

Calcium mobilization is substantially involved in platelet inside-out signalling leading to cytoskeletal re-organization and shape change, granules secretion, and integrin $\alpha_{IIa}\beta_3$ activation, as well as in subsequent $\alpha_{IIa}\beta_3$-mediated outside-in signalling.

To investigate the impact of calcium mobilization under our experimental setting we chelated intracellular calcium using BAPTA-AM (Fig. 4 A and B). When assessing platelets dense granules secretion and aggregation (Fig. 4A) we found that pretreatment with 30 μM BAPTA-AM significantly reduced ATP secretion evoked by 0.3 U/ml thrombin as well as of the combination of thrombin and 20 μM epinephrine, whereas in this setting aggregation is unaffected. The re-constituting effects of epinephrine on ATP secretion and aggregation of SNAP plus adenosine pretreated and thrombin-stimulated platelets (compare Fig. 2B), on the other hand, are obliterated by BAPTA-AM.

For (A), platelet dense granules secretion in terms of ATP release and aggregation in terms of light transmission were determined in parallel as described in “Material and Methods”. Isolated human platelets were preincubated first with vehicle (0.05% DMSO) or 30 μM BAPTA-AM for 30 min, then with both 10 μM SNAP and 10 μM adenosine (Ado) for 2 min prior to the addition of 0.3 U/ml thrombin which was followed after further 3 min by 20 μM epinephrine for another 3 min. Data of 3 individual experiments is presented as mean ± S.E.M. and statistical analysis was performed by one-way ANOVA followed by Bonferroni’s multiple comparison test. Indicated significances are compared to the effect of thrombin alone; $^{**}P \leq .01$, $^{***}P \leq .001$, ns = not significant.

In (B) flow cytometric analyses of platelet surface changes are presented. Surface expression of the secretion biomarkers LAMP-3 (Lysosome-Associated Membrane Protein 3, CD63), P-selectin (CD62P), as well as PAC-1 binding to the high-affinity conformation of integrin $\alpha_{IIa}\beta_3$, were analysed in isolated platelets as described in “Material and Methods”. Isolated human platelets were preincubated with vehicle (0.05% DMSO) or 30 μM BAPTA-AM for 30 min, followed by vehicle (0.05% DMSO) or 10 μM SNAP and 10 μM adenosine (Ado) in combination for 2 min, followed by the stimulation with 0.3 U/ml thrombin

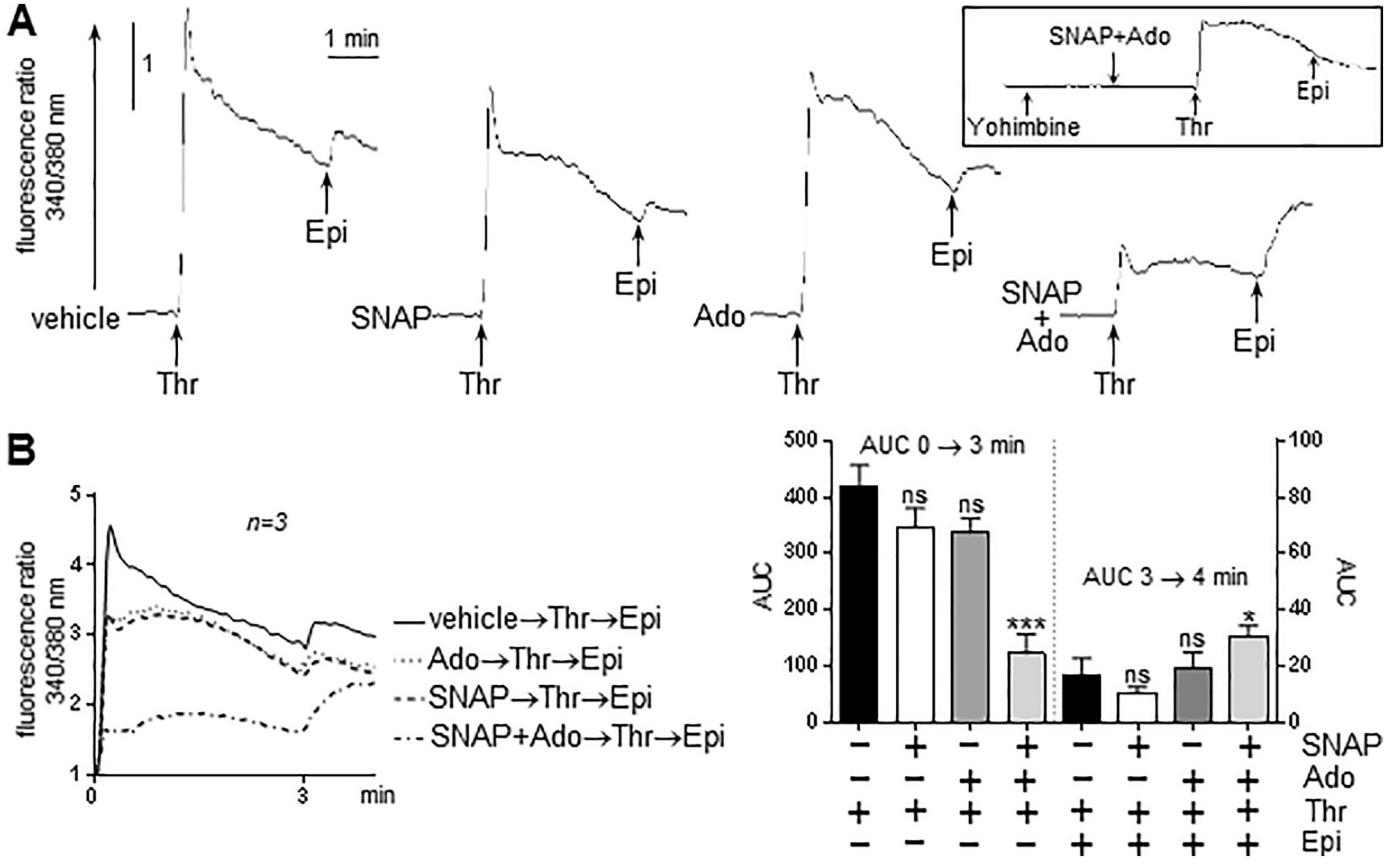

Fig. 3. The effects of elevating cGMP and cAMP cooperate in the inhibition of thrombin-evoked intracellular free calcium $[Ca^{2+}]_i$ mobilization and are counteracted by epinephrine.

(Thr) alone, or 0.3 U/ml thrombin for 3 min followed by 20 μM epinephrine (Epi) for another 3 min. Samples were analysed at time point 0 min (prior to stimulation), and 3 and 6 min after stimulation. Platelets were identified as integrin subunit α_{IIa} positive (CD41a+) cells and surface marker-positive cells are quantified as % of CD41a + cells. Data of 4 individual experiments (A) is presented as mean ± S.E.M. and statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison test; $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$, ns = not significant compared to the treatment with thrombin alone. Relevant significances for (B) are mentioned in the respective "Results" section.

Regarding the surface expression of platelet activation markers (Fig. 4B), we found that BAPTA-AM alone does not or only marginal affect the expressions of LAMP-3 or CD62P on platelets stimulated with thrombin. On the other hand, PAC-1 binding and hence the activation of integrin $\alpha_{IIb}\beta_3$ in response to thrombin is significantly reduced by BAPTA-AM from 40.13 ± 2.72% down to 21.51 ± 9.54% after 3 min of activation ($n = 3$; $P \leq .01$), and after 6 min of incubation from 33.44 ± 7.80% down to 13.76 ± 7.33% ($P \leq .001$). Regarding the reconstitutive effect of epinephrine on the expression of surface markers of platelets preincubated with SNAP and adenosine and stimulated with thrombin, we observed that the pretreatment with BAPTA-AM fully blocked LAMP-3 expression and PAC-1 binding, whereas alpha granules secretion in terms of CD62P expression was less but still significantly diminished (from 72.58 ± 7.06% to 49.72 ± 13.00%; $P \leq .05$). The latter, by contrast, was still significantly above the CD62P expression provoked by thrombin alone on SNAP and adenosine preincubated platelets (29.58 ± 14.01%, $P \leq .05$).

It may strike one peculiar that BAPTA-AM prominently dimish thrombin-provoked ATP secretion but not LAMP-3 expression. However, this may be explained by the difference in methods as mentioned above (3.2), which is continuous in the former and an endpoint measurement in the latter. Further, although PAC-1 binding in response to thrombin was found to be about 50% and significantly reduced by BAPTA-AM pretreatment, platelet aggregation appeared to be not affected, which may likewise be attributed to the various applied methods as explicated above in section 3.2. As such, even when BAPTA-AM appears to reduce PAC-1 binding in the flow cytometry assay, the number of activated integrin $\alpha_{IIb}\beta_3$ appears to be high enough for sufficient fibrinogen-binding and full platelet aggregation when assessing light transmission aggregometry.

3.5. PI3-K signalling is crucial for the reconstituting effects of epinephrine

Platelet PI3-kinase isoforms are activated by both Gαq-coupled receptors, such as the thrombin receptors PAR1 and 4, as well as, and even more prominent, by inhibitory Gα-coupled receptors, such as P2Y12 receptors and α_{2A} adrenoceptors [22,23].

As shown in Fig. 5A, in platelets preincubated with the widely applied pan-PI3-K inhibitor LY294002 at 20 μM the peak rise in secreted ATP caused by 0.3 U/ml thrombin was slightly and insignificantly reduced whereas aggregation was unaffected. The partial reconstitution of ATP secretion and full re-establishment of aggregation by 20 μM epinephrine of SNAP plus adenosine pretreated platelets stimulated with thrombin, as shown above in Fig. 2B, in contrast, was completely blocked by LY294002. When it comes to the cell surface composition (Fig. 5B) we found that LY294002 alone did not affect thrombin-provoked LAMP-3 or CD62P expression, whereas PAC-1 binding was significantly diminished from 43.84 ± 4.18% down to 24.17 ± 4.75% ($P \leq .01$) after 3 min of thrombin-stimulation, as well as from 38.14 ± 7.24% to 12.42 ± 4.64% ($P \leq .05$). The almost full reconstitution of LAMP-3 and CD62P expression due to the introduction of

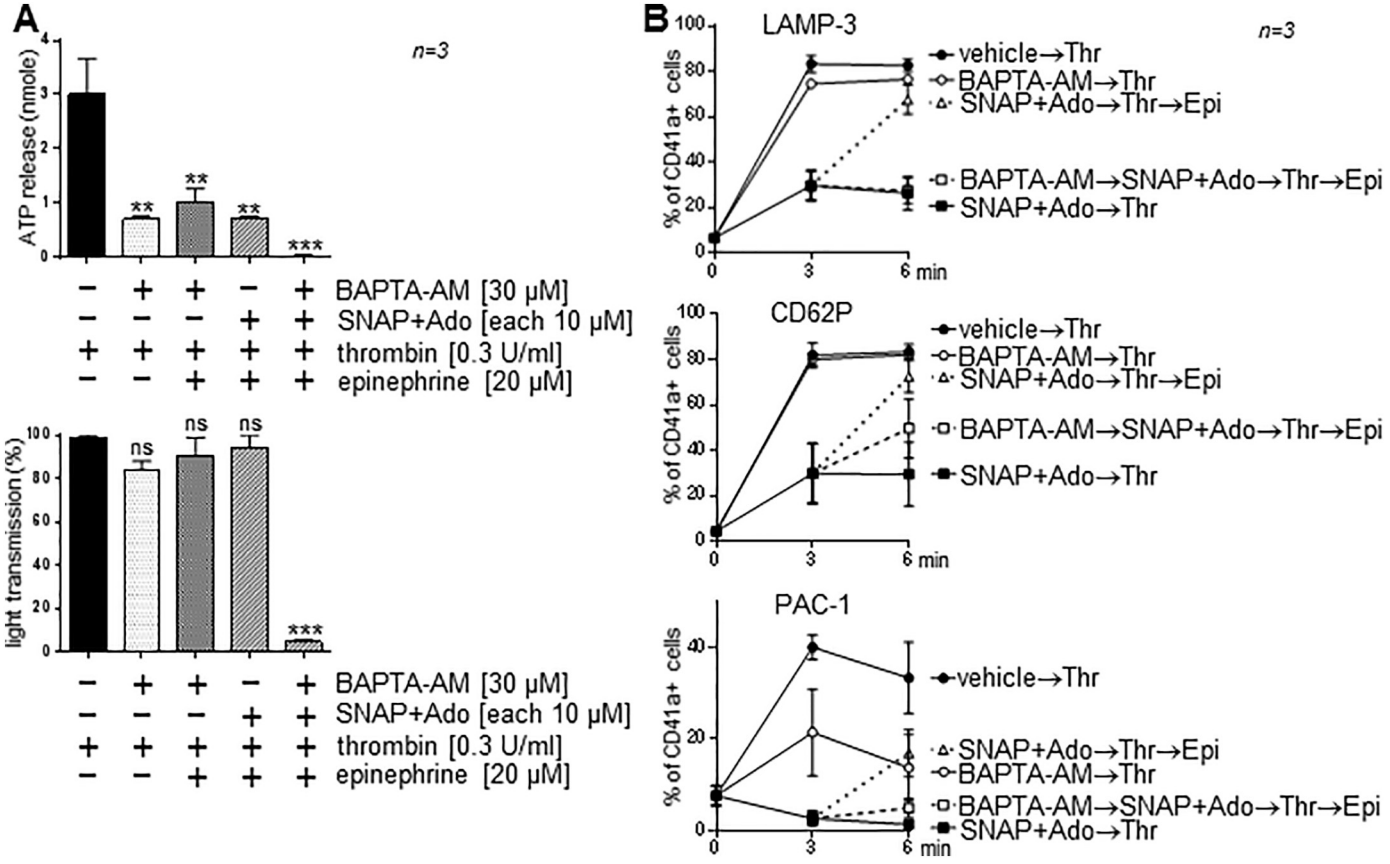

Fig. 4. The increase of intercellular $[Ca^{2+}]_i$ is pivotal for the reconstitution of dense granules secretion and platelet aggregation in response to thrombin under conditions of concomitant cGMP and cAMP elevation.

epinephrine to SNAP plus adenosine pretreated and thrombin-stimulated platelets is significantly, but not completely abrogated by LY294002 (both $P \leq .05$). Finally, and similar to the results obtained by chelating intracellular calcium with BAPTA-AM (Fig. 4B), the inhibition of PI3-K isoforms with LY294002 completely impedes the re-constituting effects of epinephrine on PAC-1 binding and therefore on the activation of integrin $\alpha_{IIb}\beta_3$. We also assessed intracellular calcium mobilization in this experimental setting (Fig. 5C). As apparent from the summary of original traces (left graph), whereas the initial peak rise in calcium mobilization initiated by thrombin is not affected by LY294002, the overall amount within 3 min of incubation is slightly but significantly affected by PI3-K isoform inhibition (right graph; AUC). However, the already prominent inhibition of the extent of thrombin-promoted calcium mobilization by SNAP and adenosine (compare also Fig. 3B), is additionally and significantly blocked by LY294002 (Fig. 5C). The impact of epinephrine on the second rise in intracellular calcium is not affected by LY294002 alone, SNAP plus adenosine (compare Fig. 3B) alone, or LY29002 in combination with SNAP plus adenosine (Fig. 5C).

For (A), platelet dense granules secretion in terms of ATP release and aggregation in terms of light transmission were determined in parallel as described in "Material and Methods". Isolated human platelets were incubated with vehicle (0.05% DMSO) or 20 µM LY294002 with gentle agitation for 30 min, then with both 10 µM SNAP and 10 µM adenosine (Ado) for 2 min prior to the addition of 0.3 U/ml thrombin which was followed after further 3 min by 20 µM epinephrine. Data of 4 individual experiments is presented as mean ± S.E.M. and statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison test; $^*P \leq .05$, $^{***}P \leq .001$, ns = not significant compared to thrombin alone. In (B) flow cytometric analyses of platelet surface changes are presented. Surface expression of the secretion biomarkers LAMP-3 (Lysosome-Associated Membrane Protein 3, CD63), P-selectin (CD62P), as well as PAC-1 binding to the high-affinity conformation of integrin $\alpha_{IIa}\beta_3$, were analysed in isolated platelets as described in "Material and Methods". Isolated human platelets were preincubated with vehicle (0.05%DMSO)) or 20 µM LY294002 with gentle agitation for 30 min, followed by vehicle (0.05% DMSO) or 10 µM SNAP and 10 µM adenosine (Ado) in combination for 2 min, followed by the stimulation with 0.3 U/ml thrombin (Thr) alone, or 0.3 U/ml thrombin for 3 min followed by 20 µM epinephrine (Epi) for another 3 min. Samples were analysed at time point 0 min (prior to stimulation), and 3 and 6 min after stimulation. Platelets were identified as integrin subunit α_{IIa} positive (CD41a+) cells and surface marker-positive cells are quantified as % of CD41a + cells. Data of 4 individual experiments (A) is presented as mean ± S.E.M. and statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison test; $^*P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$, ns = not significant compared to the treatment with thrombin alone. Relevant significances for (B) are mentioned in the respective "Results" section. For (C), rises of $[Ca^{2+}]_i$ in isolated human platelets were determined as described in "Materials and Methods". Isolated platelets were pretreated with vehicle (0.05% DMSO) or 20 µM LY294002 with gentle agitation for 30 min, then for two minutes with vehicle (0.05% DMSO), 10 µM SNAP, 10 µM adenosine (Ado), or SNAP and adenosine in combination, and then challenged with 0.3 U/ml Thrombin (Thr) followed after 3 min by 20 µM epinephrine (Epi) for 1 additional minute. The left graph shows the peak rises in intracellular $[Ca^{2+}]_i$ induced by thrombin and epinephrine, respectively, of 3 individual experiments and only the mean values are shown for reasons of lucidity. The right bar graph shows the data summarized as area under the curve (AUC) for the effects of thrombin alone within 3 min of stimulation (left part, AUC (1–3 min)) and the effects of epinephrine in the

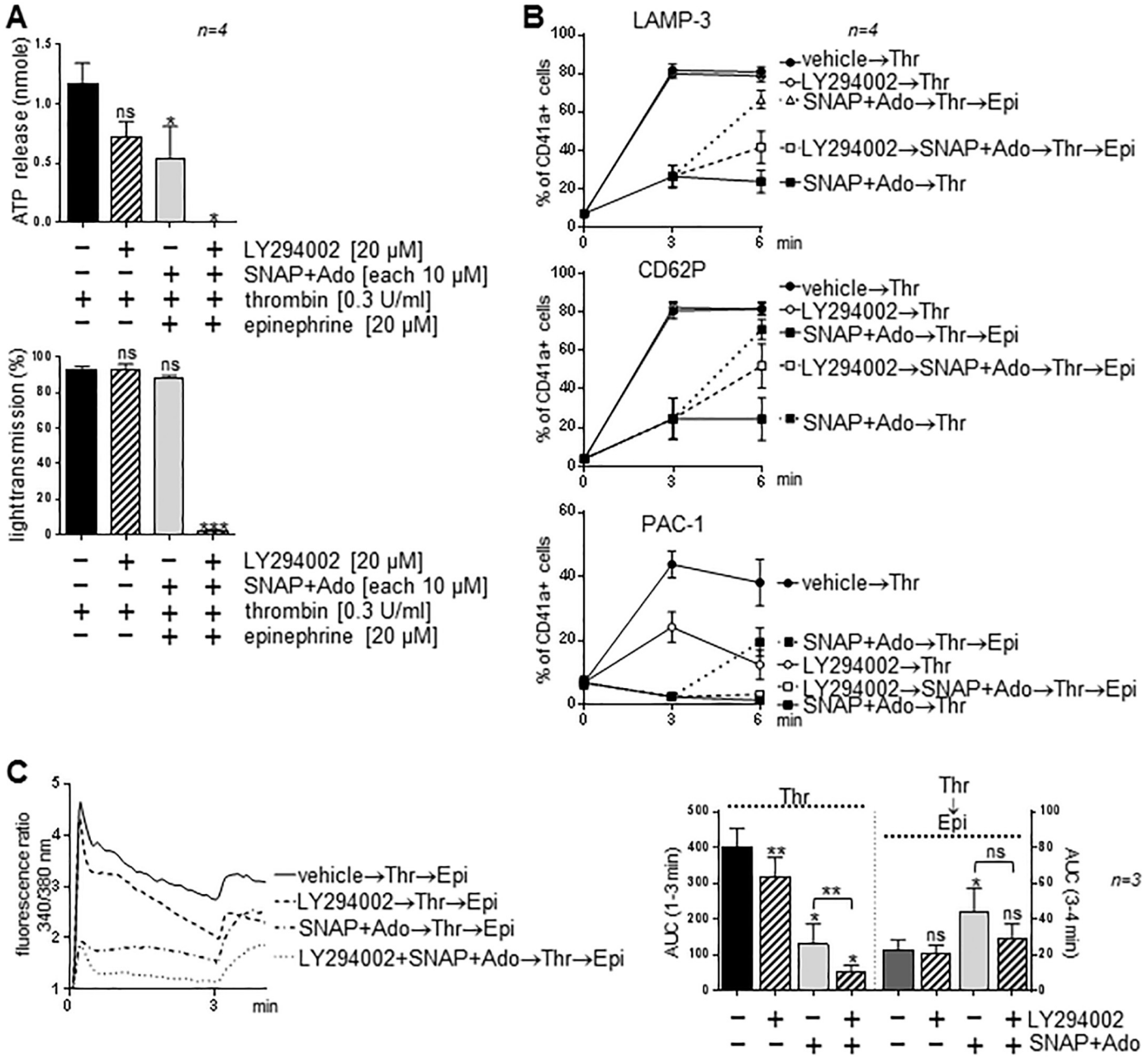

Fig. 5. PI3-K signalling is essentially involved in the reconstitution of platelet dense granules secretion and aggregation by epinephrine but not in the rise in intracellular $[Ca^{2+}]_i$.

following minute (right part, AUC 3–4 min). Statistical analysis was performed by two-tailed paired Student's *t*-tests and indicated significances are compared to thrombin alone (AUC 1–3 min) or thrombin followed by epinephrine alone (AUC 3–4 min); $*P \leq .05$, $**P \leq .01$, ns = not significant.

To evaluate intracellular signalling on a molecular level, we assessed the phosphorylation and therefore activation of the PI3-K downstream effector Akt at threonine 308 (Thr308) and serine 473 (Ser473) residues (Fig. 6). Thrombin alone at 0.3 U/ml induces massive Akt phosphorylation on Thr308 as well as Ser473, both which were less prominent but likewise significantly phosphorylated in response to 20 µM epinephrine alone. The most pronounced phosphorylation on both residues was provoked by the combination of thrombin and epinephrine.

The thrombin-provoked phosphorylation of Akt Thr308 and Ser473 was substantially blocked by pretreatment with SNAP plus adenosine. Whereas Thr308 phosphorylation is almost fully re-established by the introduction of epinephrine, that of Ser473 was to a lesser extent but still significantly reconstituted. However, these restorations were fully blocked by LY294002 and wortmannin, the latter which has been included as another commonly applied but structurally unrelated PI3-K inhibitor. Finally, neither SNAP nor adenosine, or both, or the PI3-K inhibitors LY294002 and wortmannin had any effect on basal Akt phosphorylation.

Isolated human platelets were preincubated with vehicle (0.05% DMSO), 20 µM LY294002, or 1 µM wortmannin for 30 min followed by vehicle (0.05% DMSO) or 10 µM SNAP and 10 µM adenosine in combination at 900 rpm at 37 °C for 2 min, and then stimulated with buffer or 0.3 U/ml thrombin for 3 min followed by 20 µM epinephrine for another 3 min (total 6 min). Reactions were stopped by the addition of SDS-sample buffer. Samples were analysed by SDS-PAGE followed by Western blotting as described in "Material and Methods" using

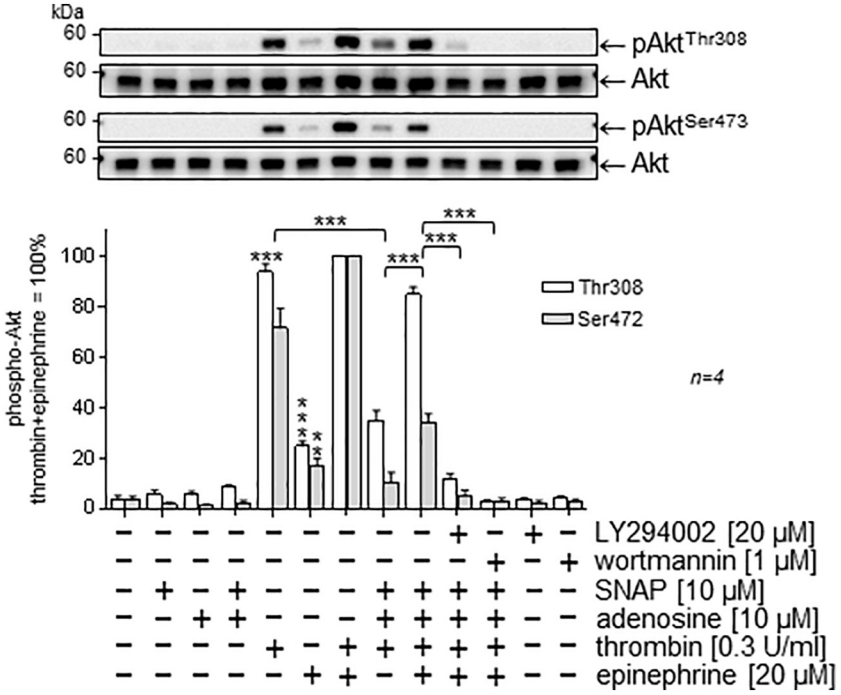

Fig. 6. The inhibitory effects of elevated cGMP and cAMP on thrombin-provoked Akt phosphorylation is bypassed by epinephrine in a PI3-K-dependent manner.

antibodies recognizing Akt phosphorylated at Thr308 residues or at Ser473 residues. Membranes were reprobed using an antibody recognizing unmodified Akt. Western blots from 4 individual experiments were densitometrically analysed and the signal induced by combined treatment with thrombin and epinephrine were set as 100%. The data is presented as mean ± S.E.M. and statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison test; $^{**}P \leq .01$, $^{***}P \leq .001$. Significances indicated without brackets relate to the unstimulated control; significance between treatments are indicated by respective brackets.

3.6. Thrombin alone or in combination with epinephrine does not modulate PKG/PKA-mediated VASP phosphorylation

The vasodilator-stimulated phosphoprotein (VASP) in its phosphorylated state is generally accepted to negatively affect platelet activation by impeding actin dynamics and integrin $\alpha_{IIb}\beta_3$ activation and signalling [2,24]. Phosphorylation kinetics indicate that Ser157 is preferentially phosphorylated as a result of cAMP elevation and PKA activation, whereas Ser239 is mainly phosphorylated via cGMP elevation and induction of PKG [25]. In addition, thrombin-receptor signalling has been shown to be able to cross-activate PKA signalling resulting in Ser157 phosphorylation [26]. As shown in Fig. 7, we likewise observed this reported pattern in our experimental setting when assessing VASP Ser157 and Ser239 phosphorylation. Further, SNAP and adenosine in combination had additive effects on the phosphorylation of both residues. Epinephrine alone had no impact on Ser239 or Ser157 phosphorylation, and also did not alter the latter provoked by thrombin. However, in platelet pretreated with SNAP plus adenosine and stimulated with thrombin, phosphorylation of Ser157 was unaltered as compared to that provoked by SNAP plus adenosine alone, and Ser239 phosphorylation was even significantly increased. In this experimental setting, subsequent addition of epinephrine did slightly but insignificantly decrease Ser157 phosphorylation, but not that of Ser239 residues. Under these conditions, however, and as above shown in for instance Fig. 2A and B, full platelet aggregation took place.

Isolated human platelets were preincubated with 10 µM SNAP, 10 µM adenosine, or both for 2 min and then stimulated with vehicle (0.05% DMSO) or 0.3 U/ml thrombin for 3 min, followed by 20 µM epinephrine for another 3 min (total 6 min). Reactions were stopped by the addition of SDS-sample buffer. Samples were analysed by SDS-PAGE followed by Western blotting as described in "Material and Methods" using antibodies recognizing (A) VASP phosphorylated at Ser157 residues or (B) VASP phosphorylated at Ser239 residues. As a loading control, membranes were reprobed using an antibody recognizing unmodified Akt. Western blots from 3 individual experiments were densitometrically analysed and the signal induced by combined treatment with SNAP and adenosine were set as 1 arbitrary unit (a.u.). The data is presented as mean ± S.E.M. and statistical analysis were performed by one-way ANOVA followed by Bonferroni's multiple comparison test; $^{**}P \leq .01$, $^{***}P \leq .001$, ns = not significant. Significances indicated without brackets relate to the unstimulated control; significance between treatments are indicated by respective brackets.

3.7. The inhibition of aggregation of platelets in whole blood by synchronous elevation of cGMP and cAMP is overcome by simultaneous PAR- and α_{2A} adrenoceptor-signalling

After investigating the molecular mechanisms in isolated platelets, we elaborated our experiments by choosing conditions more related to the in vivo environment by using whole blood (Fig. 8). For the reason of thoroughness, we also included PGI_2 in this set of experiments. Since we used heparin as anticoagulant, the latter to preclude thrombin-caused coagulation, we replaced thrombin by the protease-activated receptor (PAR)-activating peptides (PAR-APs) SFLLRN (specific for PAR1) and the AYPGKF (specific for PAR4), respectively. Both SFLLRN at 30 µM as well as AYPGKF at 300 µM induces prominent activation and aggregation of isolated platelets under our methodological conditions [27]. The PAR-APs alone, and to a lesser extent epinephrine alone, induce platelet aggregation in whole blood determined by impedance measurements. In whole blood, epinephrine does provoke human platelet aggregation, which may even be amplified by the presence of certain fractions of heparin [28]. Simultaneous elevation of cGMP with SNAP and cAMP with PGI_2 or adenosine, respectively, preclude aggregation of platelets in response to PAR-APs alone as well as to epinephrine alone. The restraining effects of elevated cyclic nucleotides are, nonetheless,

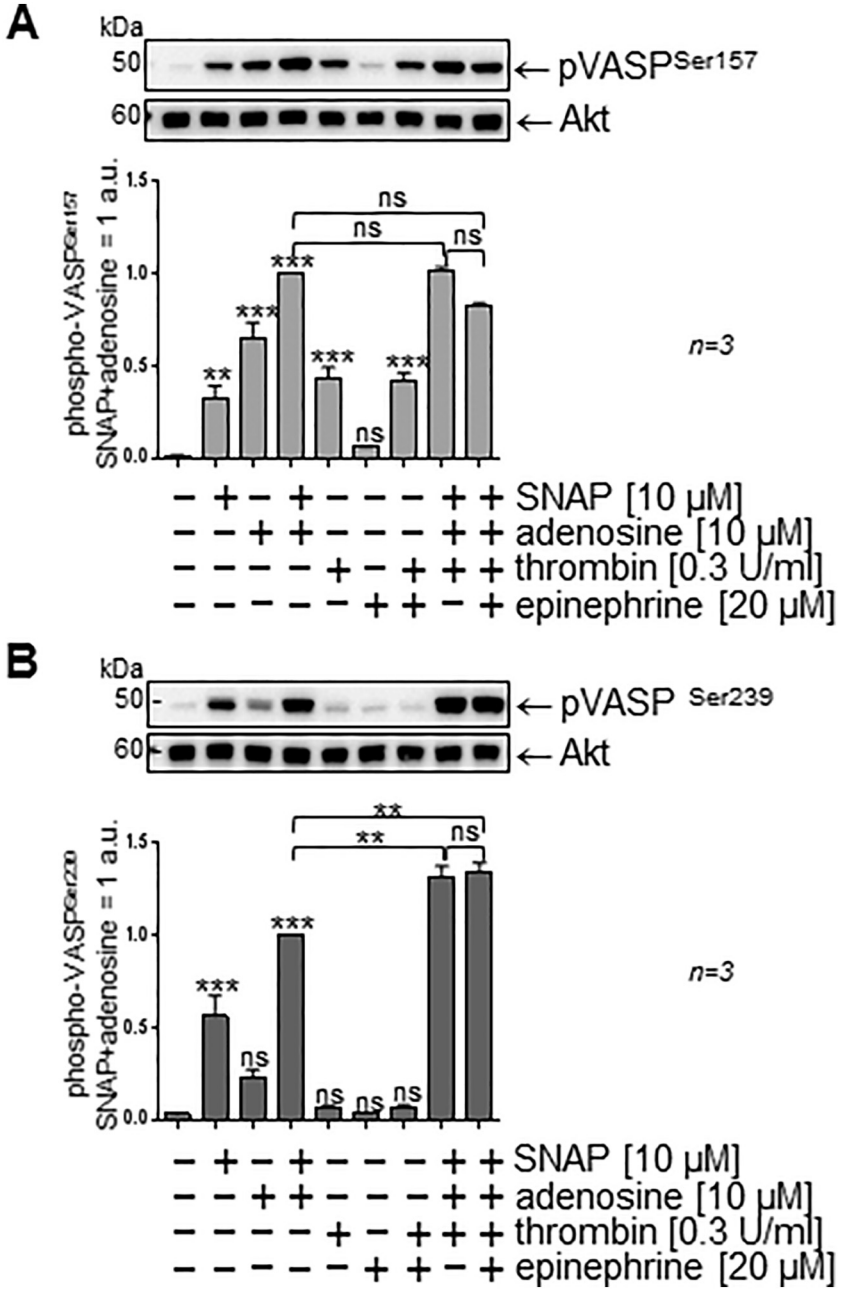

Fig. 7. Phosphorylation of platelet VASP at Ser157 and Ser239 residues as a result of elevating cGMP and cAMP but is not downregulated by sequential activation with thrombin and epinephrine.

abolished when PAR-APs and epinephrine are administered in combination.

Platelet aggregation in whole blood by impedance measurements was performed as described in "Materials and Methods". Diluted whole blood was preincubated with vehicle (0.05% DMSO), SNAP (10 µM) in combination with either PGI_2 (1 µM) or with adenosine (10 µM) for 2 min followed by the addition of PAR-activating peptides (PAR-APs) (30 µM SFLLRN plus 300 µM AYPGKF) alone, epinephrine (20 µM) alone, or PAR-APs and epinephrine in combination. Maximal platelet aggregation of 3 individual experiments is presented as mean ± S.E.M. and statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison test; $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$.

4. Discussion

The most vital innate mechanism for suppressing excessive platelet activation and thrombus formation comprises endothelial-derived autacoids which cause an increase in the levels of the intracellular cyclic nucleotides cGMP and cAMP, which in turn activate isoforms of PKGs and PKAs, respectively.

In several early studies, it has been shown that elevating cyclic nucleotides, either by inducing their generation or by preventing their breakdown, act in a synergistic manner to potently preclude human platelet activation and aggregation in response to arachidonic acid, the TXA_2-mimetic U46619, epinephrine, ADP, thrombin, as well as collagen [29–33].

This synergy may in part be explained by the observation that both PKG and PKA target several common proteins and even intramolecular phosphosites [2]. It has further been shown that NO not only causes an

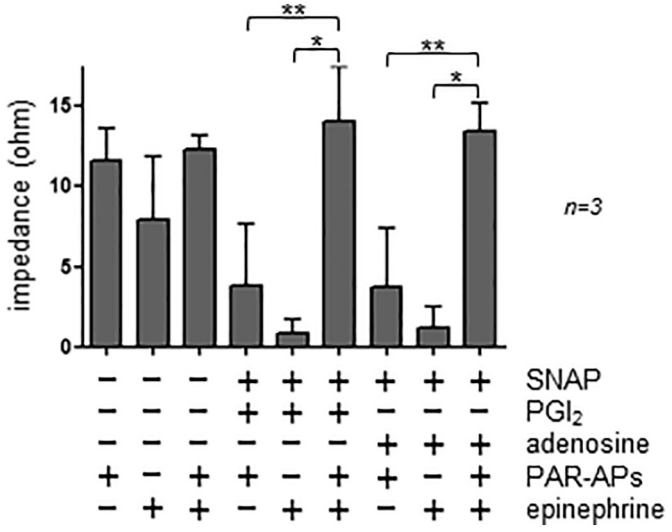

Fig. 8. The inhibitory effects of concomitant cGMP and cAMP elevation on both PAR- and α_{2A}-receptor-signalling are abolished when both receptor types are activated in parallel on platelets in whole blood.

elevation of platelet cGMP but likewise an increase in cAMP [34], the latter which has been attributed to an indirect effect by which PKG inhibits the cAMP-degrading PDE3A [35]. These concerted effects become obvious on a functional level such as platelet aggregation as well as on a molecular level when assessing Ca^{2+} mobilization or analyzing the phosphorylation of VASP, the latter for which phosphorylation kinetics have revealed that Ser157 becomes preferentially phosphorylated as a result of cAMP elevation, whereas that of Ser239 is mainly attributed to an increase in cGMP [25].

However, in more recent and very elaborate studies it has been demonstrated that P2Y12 receptor antagonists amplify to indeed remarkable extents the inhibitory effects of PGI_2 [36], of NO [37], as well as of PGI_2 combined with NO [38] on platelets which were subsequently challenged with thrombin, the PAR1-activating peptide TRAP-6, or collagen, respectively. These findings allow vice versa to conclude that P2Y12/Gαi signalling dramatically determines the inhibitory actions or potencies of cGMP/cAMP-related signalling events. In this respect, it has been demonstrated that epinephrine is able to mimic and amplify ADP-induced activation of washed human platelets after the administration of ticlopidine (which active metabolite irreversibly blocks P2Y12 receptors) even in the presence of PGI_2 [39], which is in line with our observations (Supplemental data Fig. I).

In the light of these reports, and since under our experimental setting an involvement of P2Y12 receptor signalling is absent due to the lack or marked repression of ADP secretion from dense granules, we reasoned that epinephrine-induced α_{2A} adrenoceptor signalling may be able to replace P2Y12 signalling in terms of counteracting both cGMP/cAMP-related inhibitory events. In our setting, we observed that epinephrine is indeed able to provoke platelet dense and alpha granules secretion, integrin $\alpha_{IIa}\beta_3$ activation, and aggregation via employing the adrenoceptor α_{2A} isoform, as validated by the use of yohimbine, when added prior to or subsequently after thrombin. These effects could be directly linked to α_{2A} signalling and not indirectly to the reconstitution of the release of ADP or ATP from dense granules and autocrine actions on P2Y1, P2Y12, or P2X1 receptors nor by fibrinogen-binding and integrin $\alpha_{IIb}\beta_3$ outside-in signalling (Supplemental data Fig. I).

Interestingly, when we stimulated platelets with the GPVI-agonist convulxin (Supplemental data Fig. II), epinephrine acted in a priming but not rescuing fashion. This difference may be attributed to the temporal delay between the addition of the agonists as well as varying durations of convulxin/GPVI and thrombin/PAR signalling. GPVI cross-linking by convulxin has been shown to induce rapid and transient protein-tyrosine phosphorylation in human platelets [40], whereas thrombin-induced PAR signalling consists of rapid and transient signalling via PAR1, as well as a delayed but sustained activation via PAR4 [41].

When we assessed intracellular calcium mobilization in our setting we observed a pronounced second rise of calcium mobilization induced by epinephrine, which was not mediated via PI3-K signalling downstream of Gz β/γ-subunits. This rise was more pronounced in platelets when both cGMP and cAMP was elevated compared to single treatment and may be explained by an influx of extracellular calcium by released ATP opening ionotropic P2X1 receptors and a phenomenon known as Ca^{2+}-induced Ca^{2+} release (CICR) as well as by the inhibitory action of the Gαz subunits on adenylyl cyclase [42], the latter which has been reported for the P2Y12 receptor in ADP-treated platelets [43]. The impact of released ADP and P2Y12 signalling on calcium mobilization in thrombin-stimulated platelets has been reported to rely on both the downregulation of cAMP/PKA as well as a prolonged activation of PLC by PI3-K β [44]. When we applied the widely applied pan-PI3-K inhibitor LY294002 we observed a prominent effect on Ca^{2+} mobilization provoked by thrombin, whereas that induced by epinephrine was only marginally affected, indicating that under our experimental conditions the negative regulation of adenylyl cyclase may be predominant in Ca^{2+} mobilization caused by epinephrine.

However, by interfering with either Ca^{2+}- or PI3-K-related signalling using BAPTA-AM or LY294002, respectively, we found that both are indispensable for the reconstituting effects of epinephrine on granules secretion, and especially integrin $\alpha_{IIb}\beta_3$ activation and therefore aggregation of platelets under cGMP/cAMP-elevation stimulated with thrombin.

The serine-threonine kinase Akt, also designated protein kinase B (PKB), is a well-established signalling mediator downstream of PI3-K known to be implicated in platelet granules secretion as well as integrin $\alpha_{IIb}\beta_3$ activation [45], and even a role for Akt in degrading cAMP by activating PDE3 has been proposed [46]. By assessing Akt phosphorylation on both Thr308 as well as Ser473 residues provoked by thrombin as a molecular read-out, we observed that the prominent impact of cGMP/cAMP-regulated signalling was significantly re-established by epinephrine in an LY294002- and wortmannin-dependent manner, underlining the involvement of the PI3-K/Akt-signalling cascade downstream of α_{2A} adrenoceptor activation on dense and alpha granules secretion, as well as integrin $\alpha_{IIb}\beta_3$ activation and, hence, platelet aggregation.

VASP is highly abundant in platelets and implicated in the organization of the actin cytoskeleton. Phosphorylation of VASP at Ser157 and Ser239 as a result of cAMP- and/or cGMP-elevation is generally accepted to account for precluding integrin $\alpha_{IIb}\beta_3$ activation [24].

Most of our knowledge about the role(s) of VASP derived from in vitro studies in which VASP is non-phosphorylated and cGMP/cAMP-signalling act in a competitive fashion towards Gαi-signalling. In vivo, VASP is basally phosphorylated due to the perpetual release of NO and PGI_2 from the vascular endothelium, as e.g. demonstrated for the phosphorylation of Ser157 residues [47]. The phosphorylation of Ser157 in vivo has been reported to be closely coupled to the level of NO present and correlates with platelet fibrinogen binding, alpha and dense granules secretion, as well as CD40 ligand expression [47]. In a murine VASP knock-out model, it has been shown that platelets bind more fibrinogen and aggregate faster than wild-type controls upon collagen-stimulation, and that moderate elevation of cGMP/cAMP exerts inhibitory effects only in the presence of VASP [48]. In consideration of the results obtained under our experimental setting, i.e. that epinephrine in part restored granules secretion and integrin $\alpha_{IIb}\beta_3$ activation leading to full platelet aggregation, we were indeed surprised to find that the phosphorylation of VASP was not negatively affected. However, this observation was, as lying beyond the scope of our study, not further investigated but is certainly worth further pursuit.

Platelet dense granules contain large amounts of ADP and ATP [49]

which are released on site of vascular injury upon platelet activation. Although in our experiments using isolated platelets we had to apply epinephrine at the quite high concentration to obtain a reconstituting effect in all donors, we found that in a few preparations a concentration of 200 nM epinephrine was already sufficient to fully reconstitute platelet aggregation (data not shown). However, compared to the amounts of released ADP from activated platelets, physiological plasma levels of epinephrine are comparably low, and one might argue if the effects observed under our in vitro conditions likewise may apply in vivo. Experiencing chronic stress, such as intensive care patients, has been shown to be associated with a fourfold increase of plasma epinephrine levels; and acute maximal stress during resuscitation after a cardiac arrest led to > 300-fold increase [50].

Physical stress, including intense exercise, as well as acute and chronic psychological stress, has been associated with an increased risk for thrombotic events since decades [51–53]. Although the aetiology and pathogenesis of cardiovascular diseases and acute events involve multiple and interwoven factors and mechanisms, human platelet hyperreactivity, aggregation, plug formation, vessel stenosis or occlusion surely represent major and most acute life-threatening incidents. Furthermore, mental stress not only amplifies platelet reactivity but is also associated with endothelial dysfunction [54], leading to inadequate production of endothelial autacoids which may add to the sensitivity of platelets towards activators. In this respect, and in line with the study mentioned above using platelets from volunteers after ticlopidine administration [39], a study using platelets from patients with stable coronary artery disease receiving dual antiplatelet therapy revealed that some individuals are more prone towards epinephrine-induced α_{2A} signalling than others [55]; findings that may account for one aspect of a phenomenon known as 'clopidogrel non-responsiveness'.

Taken together, and since the observations we described regarding the reconstitution of aggregation of isolated platelets also applied for platelets in heparinized whole blood ex vivo, our study may indicate that sympathetic activity during chronic stress, in concert with its negative effects on endothelial function, may prime platelets to be more susceptible towards collagen and thrombin, whereas sympathetic activity during acute maximum stress, due to for instance myocardial infarction, may amplify platelet aggregation at places of plug formation and massive thrombin-generation and might, as such, increase the extent and severity of the initial ischemic event.

Contributions

K.F., L.U.L., C.K., and M.L. designed the experiments and performed research and data analysis. A.S. provided critical input and helpful discussion and contributed to the experimental design. K.F. and M.G. interpreted results, directed the research, and drafted the manuscript; K.F. wrote the manuscript.

Conflict of interest

None to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.03.019.

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