Role of autocrine stimulation for the effects of cyclic AMP on protein and lipid phosphorylation in platelets activated by particles

Anita Ryningen*, Holm Holmsen

Department of Biochemistry and Molecular Biology, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway

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Abstract We have compared responses in platelets stimulated with the particulate materials, Intralipid (liposome-suspension) and a potential contrast medium IEEC (1′-(ethyloxycarbonyl-oxy)-ethyl-5-acetyl-amino-3-(*N*-methyl-acetylamino)-2,4,6-tri-iodo-benzenecarboxylate coated with human serum albumin), with and without forskolin and inhibitors of autocrine stimulation (IAS: an ADP-removing system of creatine phosphate/creatine phosphokinase; RGDS to prevent fibrinogen/fibronectin binding to GPIIb/IIIa; SQ 29.548 as a TXA_2 receptor antagonist; cyproheptadine as a serotonin receptor antagonist; BN 52021 as a platelet-activating factor receptor antagonist). The pattern of tyrosine-phosphorylated proteins, phosphorylation of initial lipids and phosphorylation of pleckstrin (P47) were used as markers for early signal transducing responses, while secretion of ADP+ATP and β-*N*-acetyl-glycosaminidase were used as final responses. Intralipid showed no platelet activation except for some weak tyrosine protein phosphorylation that was inhibited by elevated cAMP. IEEC induced strong platelet activation that was partly inhibited by increased levels of cAMP and IAS. The inhibition of elevated cAMP seemed to be due to removal of the G protein-mediated activation from secreted autocrine stimulators either by IAS or forskolin. The remaining activity is a pure effect from IEEC which is not affected by elevated cAMP.

Key words: Particulate agonist; cAMP; Autocrine inhibition; Platelet response

1. Introduction

Particulate contrast media of different sizes and materials have been developed to be selectively absorbed by liver and spleen in order to detect tumors in these organs [1–4]. However, platelets are activated by most foreign surfaces, and recognition of artificial (e.g. glass, gas-bubbles) and natural (collagen fibrils, bacteria, viruses and other microorganisms) particles as foreign materials, most often leads to platelet activation by ill-defined mechanisms. The IEEC contrast particles fulfil most of the criteria for a particulate contrast medium and consist of solid, iodinated (51.2 percent w/w) particles (1.28–3.57 μm in diameter) with human serum albumin coating to avoid particle aggregation [2,5].

In preliminary experiments by us IEEC was shown to aggregate human platelets, both in whole blood, PRP and GFP. The aim of this study was to elucidate the reaction mechanisms induced in human platelets by IEEC in comparison to Intralipid by measuring both early responses like protein tyrosine phosphorylation, incorporation of ^{32}P into the lipids of the polyphosphoinositide cycle (PI, PIP, PIP_2 and PA), phosphorylation of pleckstrin (P47) and final responses like secretion from the dense granules and lysosomes.

Agonist-activated platelets liberate and produce substances that are potent platelet activators: ADP, fibrinogen, fibronectin, serotonin, platelet-activating factor, TXA_2 and PGH_2. These platelet-made agonists potentiate the effects of the primary agonist in a synergistic manner [6,7]. Furthermore, close cell contact produced by platelet aggregation will also act as a potentiating stimulus [8–10]. To study the pure effects of the particles themselves, the experiments have been performed in the presence of inhibitors of autocrine stimulation (IAS): (1) creatine phosphate/creatine phosphokinase (CP/CPK) to remove ADP, (2) RGDS to prevent fibrinogen/fibronectin binding and thereby close cell contact, (3) SQ 29.548 as a TXA_2 receptor antagonist, (4) cyproheptadine as a serotonin receptor antagonist and (5) BN 52021 as a platelet-activating factor receptor antagonist. Common for these antagonists is that they act from the outside of the platelet, thus preventing the secreted products to bind to their receptors or to remove them, as in the case of ADP.

A major difference between the particulate agonist collagen and the soluble agonist thrombin is that collagen causes a massive protein tyrosine phosphorylation while thrombin does not [11,12]. Thus, protein tyrosine phosphorylation may be mandatory steps in the early signal transduction employed by the particulate agonists per se, and this has been studied in detail for IEEC and Intralipid in the present communication. Furthermore, collagen-induced responses seem to be unaffected by increased $[cAMP]_i$, in contrast to thrombin [12–14]. We wanted to determine whether IEEC and Intralipid showed the same properties as collagen by the above-mentioned criteria. We have used forskolin, which stimulates the catalytic subunit of adenylyl cyclase directly to increase $[cAMP]_i$, thus bypassing the receptor-G protein level [15,16]. Thus, gel-filtered, human platelets were treated with IEEC and Intralipid both in the absence and presence of IAS and forskolin to elucidate the activation mechanisms induced under stimulating and inhibiting conditions.

*Corresponding author. Fax: +47 (55) 58 64 00.
E-mail: anita.ryningen@pki.uib.no

Abbreviations: AHS, acid hydrolase secretion; cAMP, cyclic 3′,5′-adenosine monophosphate; $[cAMP]_i$, intracellular level of cAMP; CP, creatine phosphate; CPK, creatine phosphate kinase; DGS, dense granular secretion; ECL, enhanced chemiluminescence; GFP, gel-filtered platelets; HRP, horseradish peroxidase; IAS, inhibitors of autocrine stimulation; IEEC, 1′-(ethyloxycarbonyloxy)-ethyl-5-acetyl-amino-3-(*N*-methyl-acetylamino)-2,4,6 triiodo-benzenecarboxylate; PA, phosphatidic acid; PGH_2, prostaglandin H_2; PI, phosphatidyl-inositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidyl-inositol 4,5-*bis*phosphate; PRP, platelet-rich plasma; TXA_2, thromboxane A_2

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2. Materials and methods

2.1. Materials

[^{32}P]orthophosphate (carrier-free, Code PBS-11) was obtained from Amersham, Bucks, UK. Bovine serum albumin, fraction V (pH 7, lyophilized) was from ICN Biomedicals, Inc., Aurora, OH, USA. IEEC (1′-(ethyloxycarbonyloxy)-ethyl-5-acetyl-amino-3-(*N*-methyl-acetylamino)-2,4,6-triiodo-benzenecarboxylate) was from Nycomed Imaging A/S, Oslo, Norway, while Intralipid was from Kabi Pharmacia AB, Stockholm, Sweden. Creatine phosphate (CP), creatine phosphokinase (CPK), cyproheptadine and forskolin were purchased from Sigma, St. Louis, MO, USA, SQ 29.548 from Research Biochemicals International, MA, USA, RGDS from Calbiochem-Novabiochem Intl., La Jolla, CA, USA, BN 52021 from Biomol Research Laboratories, PA, USA. ATP monitoring reagent (a firefly luciferin/luciferase kit) was from Bio Orbit Oy, Turku, Finland. CP, CPK, RGDS and cyproheptadine were dissolved in 0.15 M NaCl, while SQ 29.548 and BN 52021 were dissolved in dimethylsulfoxide (DMSO). Stock solution of forskolin was dissolved in 96% ethanol and stored at −20°C. Stock solutions of IEEC, Intralipid, thrombin and the inhibitors of autocrine activation were diluted with their appropriate solvents to the desired concentrations just before use, and discarded after use.

2.2. Preparation of [^{32}P]P_i-labelled gel-filtered platelets

Fresh blood (450 ml), drawn into 72 ml of ACD (71 mM citric acid, 85 mM Na_3 citrate, 111 mM dextrose), was obtained at the Blood Bank, Haukeland Sykehus from healthy human volunteers who denied to have taken any medications 10 days prior to blood withdrawal. Further procedure, involving preparation of [^{32}P]P_i-labelled, gel-filtered platelets, is described elsewhere [17].

2.3. Incubations

The procedure used is described elsewhere [12]. The concentrations of the different inhibitors in the IAS solution are given in the text to Fig. 1. IEEC or Intralipid were added to the incubates at final concentrations of 10 mg I/ml and 10 mg/ml respectively, and incubated for 3 min at 37°C with stirring before aliquots were taken out for analyses.

2.4. Extraction, thin layer chromatography and determination of radioactivity of phospholipids

This was performed as described by Tysnes et al. [18] with some few modifications [17].

2.5. Tyrosine phosphorylation

The procedure used previously [11] was used without modifications. In short, proteins were detected by Western analysis of whole cell lysates using the phosphotyrosine specific antibody 4G10 and a secondary IgG antibody conjugated to HRP and visualized by ECL.

2.6. Phosphorylation of pleckstrin

IEEC caused marked [^{32}P]phosphorylation of myosin light chain (M_r = 20 kDa) and pleckstrin (M_r = 47 kDa) in the platelets (results not shown). However, elevation of intracellular cAMP by treatment of GFP with forskolin resulted in the appearance of many phosphorylated proteins with M_r in the 20–28 kDa range, as found by Haslam et al. [19], which unabled us to determine the degree of myosin light chain phosphorylation. Only pleckstrin phosphorylation was therefore determined, as described elsewhere [12].

2.7. Measurement of secreted ADP+ATP and β-N-acetylglycosaminidase

The procedures utilizing firefly luminescence for ADP+ATP and 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide as substrate for β-*N*-acetylglycosaminidases are described elsewhere [11].

2.8. Statistics

Statistical comparisons were made using Student's paired two-tailed *t*-test.

(***) $0.01 \leq P \leq 0.02$, (**) $0.02 \leq P \leq 0.05$ and (*) $P > 0.05$.

3. Results and discussion

3.1. Protein tyrosine phosphorylation

Fig. 1 (upper part) shows a comparison between IEEC- and Intralipid-induced protein tyrosine phosphorylation with re-

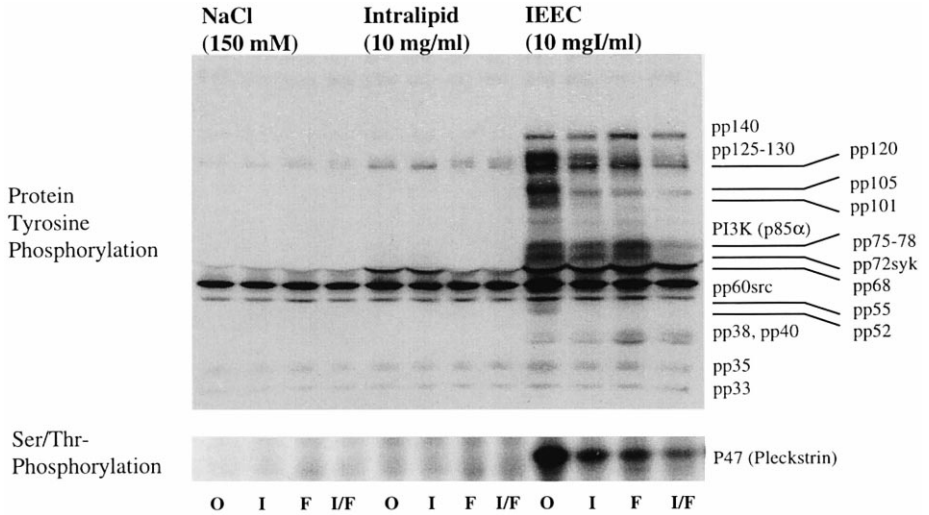

Fig. 1. Stimulation of protein tyrosine phosphorylation and [^{32}P]pleckstrin by Intralipid and IEEC, and the selective inhibition by forskolin and inhibitors of autocrine stimulation. NaCl (150 mM), Intralipid (10 mg/ml) and IEEC (10 mg I/ml) were added to 1 ml of GFP that had been treated with either solvent, inhibitors of autocrine stimulation (IAS). The volumes added and final concentrations in GFP of each autocrine inhibitor were: 20 μl of the ADP-removing system CP/CPK (5 mM/10 U/ml), 5 μl of the serotonin antagonist cyproheptadine (2 μM), 3 μl of the thromboxane A_2 receptor antagonist SQ 29.548 (150 μM), 5 μl of the platelet-activating factor (PAF) antagonist BN 52021 (150 μM) and 5 μl of the fibrinogen/fibronectin inhibitor RGDS (150 μM), forskolin (1 μM) or both forskolin and IAS. [DMSO] in the GFP was 0.77%. The IAS were preincubated in the [^{32}P]P_i-labelled gel-filtered platelets for 8 min at 37°C under non-stirring conditions, after which the particles were added and the mixtures incubated for another 3 min under stirring conditions before samples were taken out for analysis of tyrosine phosphorylation. The figure shows a Western immunoblot of proteins separated by SDS-PAGE, probed with 4G10 mouse anti-phosphotyrosine antibody and then as a secondary antibody probed with HRP-conjugated anti-mouse IgG and developed by an ECL method as described in Section 2. Lysate from 6×10^6 platelets were loaded in each lane. The results shown are representative for three independent experiments with duplicate samples. Table 1 contains the determinations of the total tyrosine phosphorylation obtained by scanning and are based on six separate immunoblots.

spect to the inhibition by forskolin and IAS. NaCl (150 mM), IEEC (10 mg I/ml) and Intralipid (10 mg/ml) were added to GFP that had been treated with either solvent, IAS alone, forskolin alone (1 μM) or both forskolin and IAS. GFP was incubated for 3 min with stirring before samples were taken out for determination of tyrosine phosphorylation. The blots were exposed optimally for the IEEC effect and are somewhat underexposed for the control samples, where proteins with molecular weight (MW) of about 33, 35, 55, 60 (src), 68 and 120 kDa were clearly phosphorylated. IAS and/or forskolin did not change this phosphorylation pattern in the control platelets. Intralipid (lane 5) induced no additional tyrosine-phosphorylated proteins, but the pp60 (src), pp68 and pp120 were more strongly phosphorylated than in the control. This pattern was not changed by addition of IAS (lane 6), whereas forskolin (lanes 7 and 8) reduced the phosphorylation of these proteins to nearly control levels. IEEC caused the appearance of many additional, intensely tyrosine-phosphorylated proteins with MW about 33, 35, 38, 40 (FcγRII), 52, 55/58 (Lyn), 60 (Src), 68, 72 (Syk), 75–78 (Cortactin), 85 (PI 3-kinase, p85α), 95/97 (Vav), pp101, pp105, pp110, pp120 (GAP), pp125 (FAK) and pp140 (PLCγ). The identity of the proteins postulated in the brackets are based on information from Clark and Brugge [20]. IAS reduced the tyrosine protein phosphorylation all over, but especially pp52, pp101, pp108 and pp125–130 were particularly reduced (lane 10). Forskolin did not reduce the tyrosine protein phosphorylation to the same degree as IAS. pp52, pp101, pp108 and pp125–130 were reduced, pp38 and pp40 were increased while the other proteins were undisturbed (lane 11). Both IAS and forskolin together, had an additive effect on tyrosine phosphorylation for pp72 (Syk), pp75–78, pp85 (p85α), pp101, pp105 and pp125–130 (lane 12).

The sum of total tyrosine protein phosphorylations (TTPP) (in percent of the total phosphorylation in control cells) obtained by scanning the films for the two agonists in the presence of IAS, forskolin or both, is given in Table 1. The phosphorylations in the O, I, F and I/F samples were calculated as percent of their respective control samples treated with NaCl. IAS did not change the Intralipid-induced TTPP significantly, suggesting that the tyrosine phosphorylations seen are due to Intralipid itself and not caused by autocrine stimulation. Forskolin alone and together with IAS reduced the phosphorylation to nearly control levels. IEEC-induced TTPP was reduced by 51 ± 7% in the presence of IAS and by 42 ± 8% with forskolin alone, suggesting that elevation of cAMP only inhibits responses to autocrine stimulation. When both IAS and forskolin were used together the TTPP was reduced by 62 ± 4%. The IEEC response is very much the same as for what is found with an other particle, collagen, and in sharp contrast to the soluble agonist thrombin [12].

3.2. Polyphosphoinositides

Intralipid caused a 132% and 30% increase in the [^{32}P]PA and [^{32}P]PI, respectively, but no significant changes in [^{32}P]PIP and [^{32}P]PIP$_2$; the increases in [^{32}P]PI and [^{32}P]PA were neither altered by IAS nor forskolin, alone and in combination (Table 1). This suggests that Intralipid does activate platelets to a small extent independently of autocrine stimulation and insensitive to elevation of cAMP. IEEC, on the other hand, gave a 18-fold increase in [^{32}P]PA (Table 1) which is substantially greater than obtained with thrombin (0.1 U/ml) and collagen (25 μg/ml) under exactly the same conditions [12]. The IEEC-induced production of [^{32}P]PA was decreased by 39 ± 11%, 53 ± 5% and 55 ± 7% in presence of IAS, forskolin and both together, respectively. This suggests that the inhibition seen is completely due to inhibition of autocrine stimulation and that forskolin has no effect on platelet activation induced by IEEC per se, except for inhibition of autocrine stimulation. The production of [^{32}P]PI, [^{32}P]PIP and [^{32}P]PIP$_2$

Table 1
Comparison between Intralipid- and IEEC-induced platelet responses with respect to their inhibition by forskolin and inhibitors of autocrine stimulation

Response	Agonist	Treatment			
		O	I	F	I+F
TTPP	Intralipid	99 ± 9	97 ± 7	15 ± 2	17 ± 2
	IEEC	996 ± 139	491 ± 67	580 ± 83	376 ± 42
PI	Intralipid	130 ± 8	133 ± 20	127 ± 11	121 ± 8
	IEEC	182 ± 21	158 ± 34	194 ± 40	174 ± 28
PIP	Intralipid	122 ± 23	114 ± 25	104 ± 12	101 ± 10
	IEEC	147 ± 8	122 ± 23	112 ± 18	102 ± 11
PIP$_2$	Intralipid	104 ± 19	114 ± 32	115 ± 23	107 ± 13
	IEEC	143 ± 21	113 ± 28	102 ± 13	97 ± 6
PA	Intralipid	232 ± 27	245 ± 16	235 ± 14	230 ± 3
	IEEC	1851 ± 196	1131 ± 245	866 ± 90	835 ± 120
P47 (pleckstrin)	Intralipid	130 ± 9	116 ± 5	110 ± 10	109 ± 12
	IEEC	533 ± 71	261 ± 44	214 ± 13	194 ± 9
DGS	Intralipid	6 ± 1	5 ± 1	6 ± 4	6 ± 3
	IEEC	92 ± 6	30 ± 6	16 ± 6	10 ± 3
AHS	Intralipid	2 ± 0	2 ± 0	2 ± 0	2 ± 0
	IEEC	24 ± 6	9 ± 1	3 ± 1	2 ± 1

GFP was treated in four different ways before stimulation with saline, Intralipid (10 mg/ml) and IEEC (10 mg I/ml). To O was added the solvent of IAS. To I was added the IAS (RGDS, CP/CPK, SQ 29.548, cyproheptadine and BN 52021) in the concentrations described in the text to Fig. 1. To F was added 1 μM forskolin and to I/F was added both IAS and forskolin. The GFP mixtures were incubated for 8 min at 37°C under non-stirring conditions with forskolin prior to addition of Intralipid or IEEC. The incubates were then stirred for 3 min before samples were taken out for analyses. The figures are given as means ± S.E. (duplicate determinations in each of three experiments) of the percentage of the control platelets without thrombin or collagen. The figures for dense granule and acid hydrolase secretion are means ± S.E. (duplicate determinations in each of three experiments) given as percent of the maximal amount secreted. Maximal secretion is obtained from stimulation of GFP with thrombin (5 U/ml) without IAS and forskolin. Fig. 1 shows one representative immunoblot of the total tyrosine phosphorylation.

in IEEC-stimulated platelets showed small and insignificant differences in the presence of IAS and/or forskolin.

3.3. Protein kinase C activation

Pleckstrin (P47) is abundant in platelets and the major substrate for PKC [21]. Again, there were no significant differences in the Intralipid-induced phosphorylation of pleckstrin alone, or in the presence of IAS and/or forskolin. IEEC produced a massive phosphorylation of pleckstrin (Table 1 and Fig. 1, lower part) which also was greater than obtained with thrombin and collagen [12]. The IEEC-induced phosphorylation was significantly inhibited by IAS (51 ± 8%) and forskolin (60 ± 4%), and, as for the TTPP and [^{32}P]PA production discussed above, we suggest that the inhibition is due to abolished autocrine stimulation and the phosphorylations seen are due to the IEEC-platelet interaction per se.

3.4. Secretion from dense granular (DGS) and lysosomes (AHS)

Intralipid did not induce significant secretion of ADP+ATP or acid hydrolase, and presence of IAS and/or forskolin made no difference (Table 1). However IEEC induced almost maximal DGS which was significantly inhibited by 67 ± 7%, 83 ± 7% and 89 ± 3%, for IAS, forskolin and both together, respectively. The AHS secretion was only 26% of the DGS and showed the same trends as for DGS concerning the effect of IAS and forskolin; the inhibition was 63 ± 4%, 88 ± 4% and 92 ± 4%, for IAS, forskolin and both together. In contrast to the other responses recorded in Table 1, the secretion response seemed to be more sensitive to forskolin. Again, there are striking similarities with collagen [12], suggesting that the inhibition seen is due to autocrine inhibition, and that the remaining effect is a pure IEEC effect.

The difference in platelet activation between Intralipid and IEEC may be explained by their difference in size and surface properties. Intralipid, consisting of hydrophile liposomes with particle diameter < 1 μm, might in addition to the anti-aggregating hydrophilicity [22], also because of their small size have a platelet-covering effect that inhibits direct platelet-platelet contact. IEEC is an albumin-coated particle with particle size from 1.28–3.57 μm [2], and is because of its size capable of activating platelets due to the recognition effect [23]. In deficiency of proteins, IEEC will be hydrophobic and activate platelets [22]. This would nearly force platelets to adhere to IEEC and give false indications of platelet activation. The albumin coat in addition to the albumin present in Tyrode's buffer should however prevent this situation.

In conclusion, the IEEC particle shows much similarities with the particulate collagen in its activation of platelets [12]. Both use tyrosine protein phosphorylation as an integral part of their signal transduction mechanism which is insensitive to elevated $[cAMP]_i$. The inhibition seen in the presence of inhibitors of autocrine stimulation, is a result of removal of the platelets self-made activators, with the remaining activity due to IEEC per se. These reaction mechanisms are very unlike from those we find with thrombin as a soluble agonist acting through G protein-mediated receptors, and might suggest a common activation pathway for particulate materials in general.

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