Anandamide activates human platelets through a pathway independent of the arachidonate cascade

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Abstract Anandamide (arachidonoylethanolamide, AnNH) is shown to activate human platelets, a process which was not inhibited by acetylsalicylic acid (aspirin). Unlike AnNH, hydroperoxides generated thereof by lipoxygenase activity, and the congener (13-hydroxy)linoleoylethanolamide, were unable to activate platelets, though they counteracted AnNH-mediated stimulation. On the other hand, palmitoylethanolamide neither activated human platelets nor blocked the AnNH effects. AnNH inactivation by human platelets was afforded by a high-affinity transporter, which was activated by nitric oxide-donors up to 225% of the control. The internalized AnNH could thus be hydrolyzed by a fatty acid amide hydrolase (FAAH), characterized here for the first time.

Key words: Anandamide; Arachidonate; Endocannabinoid; Lipoxygenase; Platelet

1. Introduction

Anandamide (arachidonoylethanolamide, AnNH) is an endogenous lipid which binds to cannabinoid receptors in the central nervous system (CB1 receptor) and in peripheral immune cells (CB2 receptor). AnNH is released from depolarized neurons [1] and mimics the pharmacological effects of Δ^9-tetrahydrocannabinol, the active principle of hashish and marijuana [2,3]. The manifold pharmacological effects of AnNH in the central nervous system (reviewed in [4]), as well as its ability to depress the inflammatory response of mast cells [5] and to damp the emerging pain signals at sites of tissue injury [6], are terminated by a rapid and selective carrier-mediated uptake into cells, followed by degradation to ethanolamine and arachidonic acid by the enzyme fatty acid amide hydrolase (FAAH).

Human platelets share several receptors and transduction signaling pathways with neuronal cells [7,8]. It has recently been shown that AnNH is present in rat blood plasma at nanomolar concentrations [9]. However, the local concentration of the lipid can be much higher, due to the local release of AnNH by macrophages [10] and endothelial cells [11]. Remarkably, AnNH decreases systemic blood pressure [12] and has been implicated in the hemorrhagic hypotension, via a CB1-mediated mechanism [10]. Moreover, AnNH acts as a vasorelaxant, sharing a common mechanism with the endothelium-derived hyperpolarizing factor [13]. Despite the growing evidence of the modulation of blood circulation by AnNH, no information is yet available on the ability of this lipid in regulating platelet function. This prompted us to investigate whether human platelets (i) were sensitive to AnNH stimulation, and (ii) were able to degrade this lipid. The effects of AnNH metabolites generated by lipoxygenase were also studied, because the activity of this enzyme has been shown to play a critical role in the platelet sensitivity to physiological agonists, such as ADP [14].

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Abbreviations: AnNH, anandamide (arachidonoylethanolamide); CB1/2, cannabinoid receptor 1/2; PaNH, palmitoylethanolamide; FAAH, fatty acid amide hydrolase; PMSF, phenylmethylsulfonyl fluoride; ASA, acetylsalicylic acid; NO, nitric oxide; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SPER/NO, spermine NONOate; (13-H)ODNHEtOH, (13-hydroxy) linoleoylethanolamide; 15-/11-H(P)AnNH, 15-/11-hydro(pero)xy anandamide; PBS, phosphate-buffered saline; RP-HPLC, reversed phase high performance liquid chromatography; GAR-AP, goat anti-rabbit alkaline phosphatase conjugate

2. Materials and methods

2.1. Materials

Chemicals were of the purest analytical grade. Anandamide (arachidonoylethanolamide, AnNH), arachidonic acid, palmitic acid, ethanolamine, ADP, phenylmethylsulfonyl fluoride (PMSF), sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Spermine NONOate (SPER/NO, (Z)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino}-diazen-1-ium-1,2-diolate) was from Alexis Corporation (Läufelfingen, Switzerland). Fluo-3/AM was obtained from Molecular Probes (Eugene, OR, USA). [1-^{14}C]AnNH was synthesized from ethanolamine and [1-^{14}C]arachidonic acid (52 mCi/mmol, NEN, Boston, MA, USA) as reported [15]. Palmitoylethanolamide (PaNH), linoleoylethanolamide (9*Z*,12*Z*-octadeca-9,12-dienoylethanolamide, ODNHEtOH) and the 13-hydroxy derivative 13-HODNHEtOH were synthesized and characterized (purity > 96% by gas-liquid chromatography) as reported [16]. 15-Hydro(pero)xyanandamide (15-hydro(pero)xyeicosa-5*Z*,8*Z*,11*Z*,13*E*-tetraenoylethanolamide, 15-H(P)AnNH, purity > 96%) and 11-hydro(pero)xyanandamide (11-H(P)AnNH, a mixture of 45% 11-H(P)AnNH, 24% 5-H(P)AnNH, 18% 15-H(P)AnNH, 9% 8-/9-H(P)AnNH and 4% 12-H(P)AnNH by reversed phase high performance liquid chromatography) were synthesized as described [17].

2.2. Preparation of human platelets

Blood was drawn from healthy donors who had not taken any drugs for at least one week before the donation. Blood was collected into anti-coagulant citrate-dextrose solution (Sigma Chemical Co., St. Louis, MO, USA), then platelet-rich plasma and washed platelets were prepared as described previously [18]. Platelets activation was monitored by the change in light transmission at 650 nm in a model 840 dual-channel Elvi aggregometer (Elvi Logos, Milan, Italy) [19]. Cytosolic calcium concentrations were determined by Fluo-3/AM fluorescence, as reported [20]. Dye loading was achieved by incubating washed platelets (10×10^6/ml) in 20 μM Fluo-3/AM dissolved in calcium and magnesium-free phosphate-buffered saline (PBS), for 45 min

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at 37°C. Afterwards, platelets were washed twice in PBS and resuspended in Hank's balanced salt solution (Flow Laboratories Ltd., Herts, UK). Fluorescence was measured in a FACSCalibur cytofluorimeter (Becton-Dickinson, CA, USA). Platelet morphology was analyzed by standard cytofluorimetric procedures.

2.3. Characterization of fatty acid amide hydrolase (FAAH)

Washed platelets (4×10^9) were collected in phosphate-buffered saline and centrifuged at $1000\times g$ for 10 min. The dry pellet was resuspended in 1 ml ice-cold 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA (buffer A) and sonicated on ice three times for 10 s, with 10 s intervals, using a Vibracell sonifier (Sonics and Materials Inc., Danbury, CT, USA) with a microtip at maximum power. Membranes from platelet homogenates were then prepared as described [21,22] and the final pellet, containing most FAAH activity [23,24], was resuspended in 100 μl ice-cold buffer A at a protein concentration of 1 mg/ml and stored at −80°C until use.

FAAH (E.C. 3.5.1.4, arachidonoylethanolamide amidohydrolase) activity was assayed by reversed phase high performance liquid chromatography (RP-HPLC), as described [15]. Kinetic studies were performed using different concentrations of [1-^{14}C]AnNH (in the range 0–30 μM). Kinetic parameters were calculated by fitting the experimental points to a Lineweaver-Burk plot with a linear regression programme (Kaleidagraph 3.0). Straight lines with r values >0.95 were obtained. The effect of various compounds on the hydrolase activity of FAAH was determined by adding directly each substance to the assay buffer, at the indicated concentrations.

Immunochemical analysis of human platelet FAAH was performed by SDS-polyacrylamide gel electrophoresis (12%), under reducing conditions, in a Mini Protean II apparatus (Bio-Rad, Richmond, CA, USA) with 0.75 mm spacer arms [25]. Rainbow molecular weight markers (Amersham, Buckinghamshire, UK) were phosphorylase b (97.4 kDa), bovine serum albumin (66.0 kDa) and ovalbumin (46.0 kDa). Platelet homogenates (20 μg/lane), prepared as described above for FAAH assay, were subjected to SDS-polyacrylamide gel electrophoresis, then slab gels were electroblotted onto 0.45 μm nitrocellulose filters (Bio-Rad), using a Mini Trans Blot apparatus (Bio-Rad) as reported [25]. Immunodetection of FAAH on nitrocellulose filters was performed with specific anti-FAAH polyclonal antibodies (diluted 1:200), elicited in rabbits against the conserved FAAH sequence VGYYETDNYTMPSPAMR [26], conjugated to ovalbumin. This peptide antigen and the anti-FAAH polyclonal antibodies were prepared by Primm S.r.l. (Milan, Italy). Goat anti-rabbit alkaline phosphatase conjugate (GAR-AP, Bio-Rad), diluted 1:2000, was used as second antibody and immunoreactive bands were stained with the alkaline phosphatase staining solution according to the manufacturer's instructions (Bio-Rad).

2.4. Determination of anandamide uptake

The uptake of [1-^{14}C]AnNH (52 mCi/mmol) by intact platelets was studied essentially as described [23], using 2×10^8 platelets in 2 ml phosphate-buffered saline (PBS) in each test. Suspensions of human platelets were incubated for 20 min at 37°C with [1-^{14}C]AnNH in the 0–1000 nM range, then they were washed three times in PBS containing 1% bovine serum albumin and were finally resuspended in 200 μl PBS. Membrane lipids were then extracted [27], resuspended in 0.5 ml methanol, mixed with 3.5 ml Sigma-Fluor liquid scintillation cocktail for non-aqueous samples (Sigma), and radioactivity was measured in a LKB1214 Rackbeta scintillation counter (Sweden). Control experiments were carried out at 4°C, in order to check whether AnNH import into cells took place by facilitated transport or by simple diffusion [23]. Q_{10} value was calculated as the ratio of AnNH uptake at 30°C and 20°C [28]. Apparent K_m and V_{max} values of the uptake kinetics were calculated by Lineweaver-Burk analysis (in this case, the uptake at 4°C was subtracted from that at 37°C). AnNH uptake was expressed as pmol AnNH taken up per min per mg protein. The effect of different compounds on AnNH uptake was determined by adding each substance directly to the incubation medium, at the indicated concentrations.

2.5. Data analysis

Data reported in this paper are the mean (±S.D.) of at least three independent determinations, each in duplicate. Statistical analysis was performed by the Student's t-test, elaborating experimental data by means of the InStat programme (GraphPad Software).

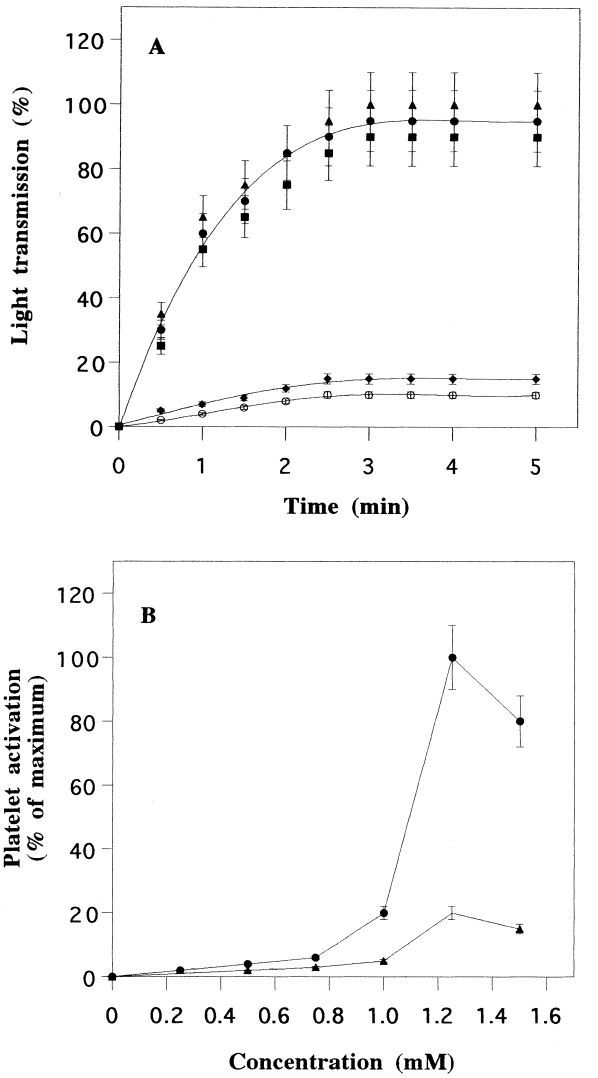

Fig. 1. A: Washed platelets were treated with 1.3 mM AnNH, alone (circles) or in the presence of 100 μM PMSF (triangles), 100 μM ASA (squares), 1.3 mM 11-H(P)AnNH (diamonds) or 1.3 mM ODNHEtOH (empty circles). B: Dose-dependent activation of platelets by AnNH (circles) or PaNH (triangles).

3. Results

Washed platelets were activated by 1.3 mM AnNH, a process which reached a maximum 3 min after addition of the lipid (Fig. 1A). Activation of platelets by AnNH was superimposable to that by arachidonate at the same concentration (not shown), though it was not affected by co-incubation with either 100 μM PMSF or 100 μM ASA (aspirin) (Fig. 1A). Unlike AnNH, its hydro(pero)xy derivatives 11-H(P)AnNH and 15-H(P)AnNH, and the congeners ODNHEtOH and 13-HODNHEtOH, did not activate human platelets, but they abolished the effect of AnNH when added together at the same concentration (Fig. 1A). Platelet activation by AnNH was dose-dependent, reaching a maximum at 1.3 mM (Fig. 1B). Instead, palmitoylethanolamide (PaNH) was unable to activate platelets in the same concentration range (Fig. 1B) and did not interfere with the effect of AnNH (data

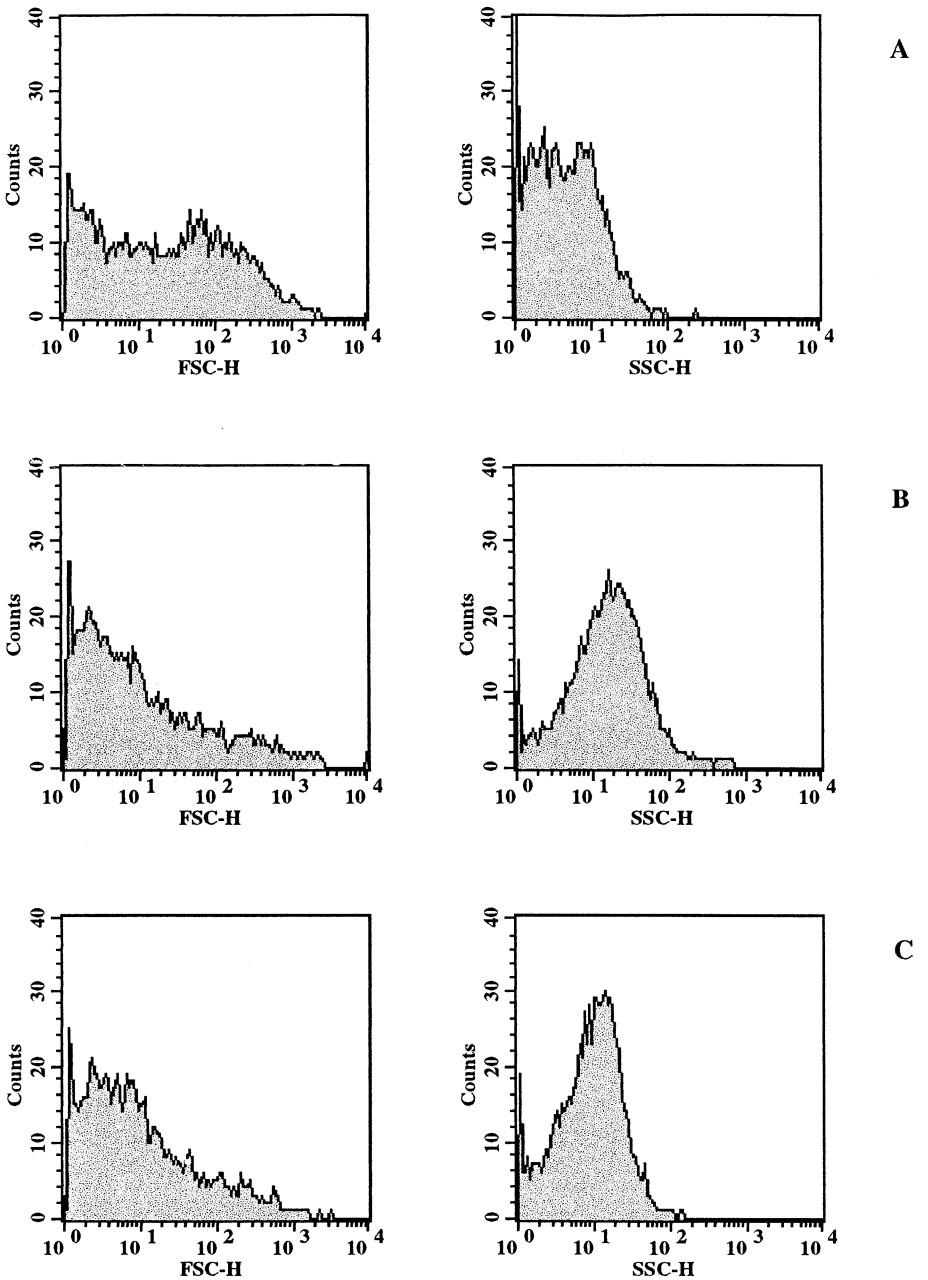

Fig. 2. Washed platelets, resting (A) or stimulated for 1 min with 1.3 mM AnNH (B) or arachidonic acid (C), were analyzed by cytofluorimetry for cell size (forward scatter-height, FSC-H) and complexity (side (90°) scatter-height, SSC-H).

not shown). Platelet activation by millimolar concentrations of AnNH was not observed in platelet-rich plasma, while it afforded the 'ADP priming', i.e. it was able to make irreversible platelet activation induced by 0.2 μM ADP (data not shown).

The effects of AnNH and arachidonic acid on the overall morphology of washed platelets (i.e. cell size and complexity) under the same conditions were superimposable (Fig. 2). Like arachidonic acid, the effect of AnNH on platelets could be attributed to a remarkable increase in cytosolic calcium concentration, which again was not affected by either 100 μM PMSF or 100 μM ASA (Fig. 3A). Unlike AnNH, its derivatives 11-H(P)AnNH and 15-H(P)AnNH, and the congeners ODNHEtOH and 13-HODNHEtOH, caused only a modest increase of cytosolic calcium concentration (Fig. 3B), which was even less upon treatment of platelets with PaNH (Fig. 3C).

Intact platelets were able to accumulate [^{14}C]AnNH, a process which was temperature- ($Q_{10} = 1.5$), time- ($t_{1/2} =$ 5 min) and concentration-dependent (Fig. 4A and data not shown). [^{14}C]AnNH uptake was saturable ($K_m = 0.20 \pm 0.02$ μM, $V_{max} = 22 \pm 2$ pmol min^{-1} mg protein^{-1}) at 37°C. The uptake was enhanced when the incubations were carried out in the presence of NO-donors such as SNP (Fig. 4B), SNAP or SPER-NO (not shown). These donors were used at millimolar concentrations which release physiological nanomolar concentrations of NO in solution [29,30]. Enhancement of [^{14}C]AnNH uptake by SNP was prevented by the presence of hemoglobin (Fig. 4B), a typical NO scavenger [31]. Conversely, the alkylating agents PMSF, iodoacetic acid or *N*-

ethylmaleimide, each used at 100 μM final concentration, reduced AnNH uptake by platelets to approximately 40% of the untreated control (data not shown). Finally, 10 μM arachidonic acid, 11-H(P)AnNH, 15-H(P)AnNH, ODNHEtOH or 13-HODNHEtOH did not affect the [^{14}C]AnNH uptake by intact platelets (not shown).

Platelets also showed a remarkable FAAH activity, which hydrolyzed AnNH in a Michaelis-Menten way (Fig. 5A). Lineweaver-Burk plot of FAAH-catalyzed hydrolysis of AnNH allowed to calculate an apparent $K_m = 10 \pm 1$ μM and $V_{max} = 270 \pm 30$ pmol min^{-1} mg protein^{-1}. Western blotting of platelet homogenates (20 μg/lane) showed that anti-FAAH polyclonal antibodies specifically recognized a single immunoreactive band, corresponding to a molecular weight of approximately 67 kDa (Fig. 5B). The alkylating agents PMSF, iodoacetic acid or *N*-ethylmaleimide, each used at 100 μM final concentration, almost completely inhibited FAAH (data not shown). The same inhibition and electrophoretic patterns have been recently shown in human brain FAAH [32].

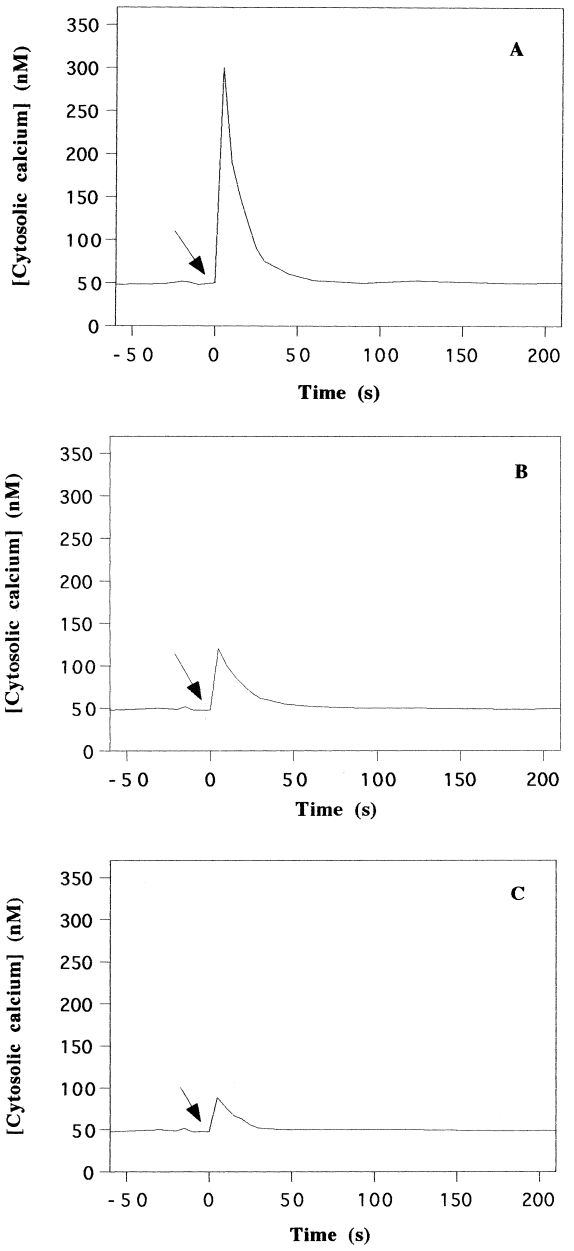

Fig. 3. Intracellular calcium concentration was measured in washed platelets, upon treatment with A: 1.3 mM AnNH; B: 1.3 mM 11-H(P)AnNH, 15-H(P)AnNH, ODNHEtOH or 13-HODNHEtOH; C: 1.3 mM PaNH. Treatment of platelets with 1.3 mM AnNH in combination with 100 μM PMSF or ASA yielded the same results as treatment with 1.3 mM AnNH alone (panel A). Arrows indicate the addition of each compound to the platelet suspension.

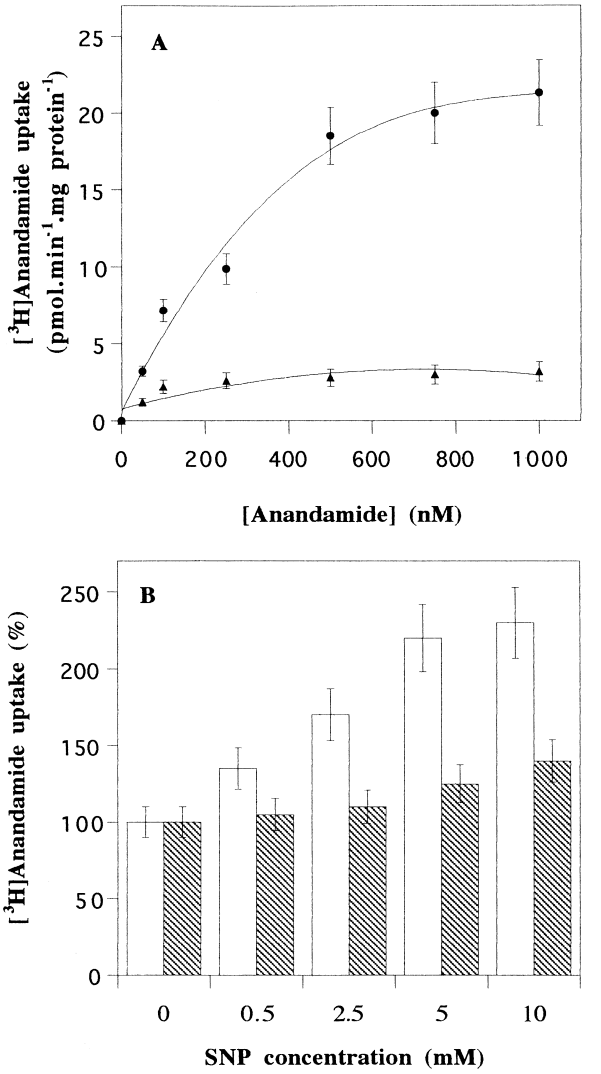

Fig. 4. A: Dependence of [^{14}C]AnNH uptake by human platelets (20 min) on AnNH concentration, at 37°C (circles) or 4°C (triangles). B: Effect of nitric oxide-donor SNP, alone (empty bars) or in combination with 20 μM hemoglobin (hatched bars) on the uptake of 200 nM [^{14}C]AnNH. Uptake was expressed as percentage of the untreated control (100% = 10 ± 1 pmol min^{-1} mg protein^{-1}).

4. Discussion

Platelets are small disc-shaped cell fragments which undergo a rapid transformation upon vascular damage. They soon become more spherical and extrude pseudopodia, eventually forming a plug which is responsible for primary hemostasis. Activation of platelets is also implicated in the pathogenesis of unstable angina, myocardial infarction and stroke [33]. Plate-

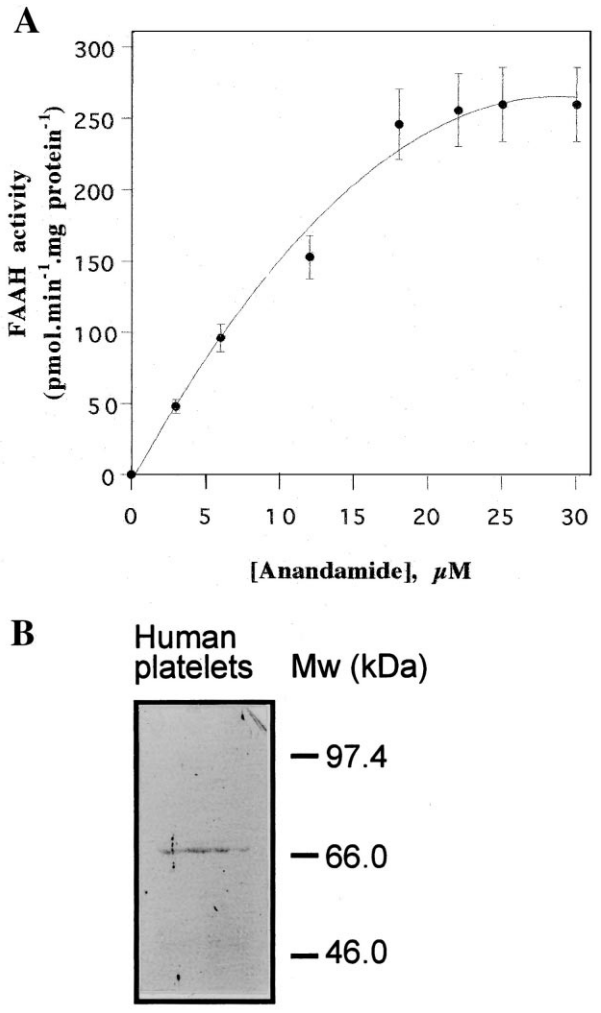

Fig. 5. A: FAAH activity was assayed by incubating 20 μg platelet homogenate with different amounts of anandamide, for 15 min at 37°C. B: Western blot analysis of platelet homogenates (20 μg/lane), separated by 12% SDS-PAGE and reacted with specific anti-FAAH polyclonal antibodies.

lets can be also stimulated 'in vitro' by physiological agonists such as arachidonic acid and ADP, which induce a shape change of platelets, from smooth discs to spiculated spheres, with emission of pseudopodia which may lead to their aggregation [7,8,14]. A common metabolic response of platelets to these agonists is the increase in cytosolic calcium [7,8,14].

Hemorrhagic shock [10], and more in general peripheral tissue injury [12], can be induced by anandamide, which is present in blood plasma at nanomolar concentrations [9] but if released locally by macrophages and endothelial cells it can reach even higher concentrations [10,11]. We have found that AnNH much alike arachidonic acid can activate human platelets, with a similar time-course and at the same concentration (Fig. 1A). Furthermore, AnNH induces the same morphological changes (Fig. 2B and C) and increases intracellular calcium concentration as arachidonic acid (Fig. 3A and data not shown). However, AnNH-induced activation of platelets was insensitive to ASA (Figs. 1A and 3A), which abolishes the effect of arachidonic acid through the inhibition of cyclooxygenase [34], thus preventing the formation of the potent platelet agonist thromboxane A_2. Moreover, PMSF, which inhibits the FAAH-catalyzed release of arachidonate from AnNH, did not affect platelet activation by AnNH (Fig. 1A), supporting the hypothesis that AnNH acts through a pathway independent of arachidonate metabolism.

The interaction of human platelets with AnNH derivatives was further characterized, because lipoxygenase activity of platelets, mainly due to a 12-lipoxygenase isozyme, has been shown to play a critical role in modulating platelet response to physiological stimuli [14]. AnNH hydro(pero)xides, which are produced by lipoxygenases [35], were unable to activate human platelets, but prevented the activation by AnNH (Fig. 1A). A similar effect was observed with linoleoylethanolamide, normally present in neurons [1], and with the 13-hydroperoxide generated thereof by lipoxygenase (Fig. 1A). Instead, palmitoylethanolamide, whose activity in mast cells [5] and skin [6] is antagonized by AnNH, did not show any effect on platelets (Fig. 1B) nor antagonized AnNH (not shown). This evidence and the observation that AnNH was active only at millimolar concentration (Fig. 1B), seem to rule out a CB1 or CB2 receptor-mediated platelet activation. The nature of AnNH target on platelet surface remains to be elucidated, but it is well known that the platelet stimulus-response coupling is often very complex. For instance, ADP has been known to activate platelets since 1961, but only recently it has been shown that four different receptors, each coupled to a different signaling pathway, are required for its action [7,8]. Even though AnNH did not activate platelets under more physiological conditions, i.e. in platelet-rich plasma, the observed synergistic effect of this lipid on ADP-induced platelet activation may suggest a physiological role for this compound.

That AnNH might be an important signal molecule for human platelets is also suggested indirectly by the observation that these cells possess the machinery to degrade this lipid. Indeed, human platelets showed a remarkable FAAH activity, which was attributable to a single protein of 67 kDa (Fig. 5). This molecular weight was in good agreement with the full-length human liver FAAH cDNA [26]. The catalytic and molecular properties of platelet FAAH are close to those observed in human brain and human neuronal (CHP100) and immune (U937) cell lines [32]. In every case, FAAH activity was inhibited by alkylating agents PMSF, iodoacetic acid and *N*-ethylmaleimide, suggesting that a cysteine residue might be critical for enzyme activity [36].

In order to be hydrolyzed by FAAH, AnNH has to be imported into the cell. Recent experiments performed on rat neuronal cells [28,37], rat basophilic leukemia (RBL-2H3) cells and mouse J774 macrophages [23] clearly showed the presence of a high-affinity AnNH transporter in the cell outer membranes. A similar methodology was used here to characterize the AnNH uptake by human platelets. These cells rapidly took up AnNH ($t_{1/2} = 5$ min), in a temperature-dependent ($Q_{10} = 1.5$) and saturable way (Fig. 4A and data not shown). The affinity of the transporter for AnNH in human platelets was comparable to that of rat astrocytes ($K_m = 0.32$ μM) [37]. Furthermore, the uptake of AnNH by human platelets was unaffected by arachidonic acid, AnNH derivatives and congeners, leukotriene B_4 or prostaglandin E_2 (not shown), suggesting that AnNH accumulation is selective and mediated by a transporter different from the long chain fatty acid transport protein [38] or the prostaglandin transporter [39]. This finding is in keeping with recent data on the AnNH carrier of rat neurons and astrocytes [37] and human cells in culture [32].

As reported for other human cells, AnNH uptake by platelets was increased by NO-donors SNP (Fig. 4B), SNAP or SPER/NO (not shown). NO is a vasodilator with anti-aggregating properties, which is released by human endothelial cells, granulocytes and monocytes upon stimulation of opiate and cannabinoid receptors [40]. The NO-mediated enhancement of AnNH uptake by platelets is coherent with the anti-aggregating properties of this molecule, because a faster AnNH removal from the extracellular space, followed by hydrolysis inside platelets, should decrease the concentration of AnNH, thus preventing platelet aggregation. On the other hand, alkylating agents PMSF, iodoacetic acid and *N*-ethylmaleimide (not shown) reduced AnNH uptake down to approximately 50% of the control, suggesting the presence of a cysteine residue in the active site of the transporter. The alkylation of this residue might reduce the velocity of AnNH import, while a nitrosylation seems to have the opposite effect.

In conclusion, the results reported here show that human platelets can be activated by AnNH, in a way independent of the arachidonate cascade. They also bring information on human platelet FAAH and AnNH transporter. In addition, they suggest that the hydro(pero)xides of arachidonate and linoleate ethanolamides generated by lipoxygenase activity might be important natural antagonists of human platelet aggregation.

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