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Activation of rabbit blood platelets by anandamide through its cleavage into arachidonic acid

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Abstract Anandamide (ANA), a cannabinoid receptor ligand, stimulated platelet aggregation at concentrations similar to those of arachidonic acid (AA). The aggregating effect of ANA was inhibited by aspirin but not by SR-141716, a cannabinoid receptor antagonist. In addition, HU-210, a cannabinoid receptor agonist, failed to induce platelet activation. Radiolabelling experiments showed that exogenous ANA was cleaved by platelets into AA through a phenylmethylsulfonyl fluoride (PMSF)-sensitive pathway. In agreement, PMSF was shown to abolish the aggregating effect of ANA. In conclusion, ANA is able to induce platelet activation via its cleavage by a PMSF-sensitive amidase activity, leading to the release of AA which in turn activates platelets.

Key words: Cannabinoid ligand; Platelet activation; Amidase activity

1. Introduction

Anandamide (ANA), i.e. *N*-arachidonoyl-ethanolamine, identified as a brain arachidonic acid (AA) derivative in various mammalian species, is a ligand for endogenous cannabinoid receptors [1–4]. Two major cannabinoid receptors have been characterized: (i) the central cannabinoid receptor CB1 was first discovered in brain and more recently also found to be present in testis and peripheral tissues [5–8]; (ii) the 'peripheral' cannabinoid receptor CB2 is predominantly expressed in peripheral tissues, and particularly in cells of the immune system [9,10]. When administered in vivo, ANA induces antinociception, hypothermia, hypomotility and catalepsy [11–14]. ANA may also be involved in pain initiation [15] or sleep induction [16]. Recent studies have suggested that ANA may be involved in immune [7,17,18], cardiovascular [4,19] or reproductive functions [20,21]. However, the effect of ANA on isolated cells implicated in these processes is poorly documented.

In brain, AA may be produced from ANA through the action of a calcium-independent specific amidase which belongs to the serine protease family, sensitive to phenylmethylsulfonyl fluoride (PMSF) [22–25]. It has been reported that ANA is stable in plasma, suggesting that the short half-life of ANA after administration depends on its degradation by cells. The presence of an amidase activity in cell types other than neurons has been demonstrated for U937 cells [25] and leukocytes [26]. However, whether this amidase is present in blood platelets and mediates a possible effect of ANA remains to be investigated.

The proaggregant action of AA has been well established, since its metabolism by cyclooxygenase and lipoxygenases leads to the formation of various biologically active AA derivatives such as thromboxane A_2 (TxA_2), and prostaglandins (PG) G_2, H_2, and E_2 [27–31]. Because ANA has an unmodified arachidonate backbone, it has been examined whether it is a substrate for the enzymes that metabolize AA. It has been shown in vitro that ANA is a substrate for porcine leukocyte and human platelet purified 12-lipoxygenases, as well as for rabbit reticulocyte and soybean 15 purified lipoxygenases [32]. Hampson et al. [33] have also demonstrated that brain cells produce 12-hydroxyanandamide, an ANA-derived compound resulting from the 12-lipoxygenase activity. Moreover, purified human cyclooxygenase 2 is able to transform ANA in PGE_2 ethanolamine [34].

It is of interest to examine the effect of ANA on blood platelets in order to determine if this compound may exert some pharmacological actions on this cell type. Theoretically, ANA might be able to induce platelet activation by three different mechanisms: (i) after cleavage by cyclooxygenase or lipoxygenase, (ii) after cleavage by an amidase leading to the production of AA, and (iii) after direct binding to cannabinoid-like receptors. Our study clearly shows that ANA induced rabbit platelet activation that correlated with its cleavage into AA by a PMSF-sensitive amidase. We suggest that ANA is a precursor of AA and derivatives, and thus may play a role as a platelet aggregating agent.

2. Materials and methods

2.1. Materials

Thrombin was from Hoffman-La Roche (Switzerland) and collagen and platelet activating factor (PAF) were from Bachem (Switzerland). Fibrinogen (grade L) was purchased from Kabi (Stockholm, Sweden) and treated with diisopropyl fluorophosphate to inactivate pro-activating contaminants. Aspirin was from Sanofi-Synthelabo (France), AA and ANA from Sigma Chemical Co. (St. Louis, MO, USA). Compounds HU-210 and SQ-29548 were purchased from Tebu (France). Compound SR-141716 was kindly provided by M. Mosse from Sanofi-Synthelabo (France).

2.2. Preparation of washed rabbit platelets

Male rabbits (HY/CR strain from Charles River, Saint-Aubin-lès-

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Abbreviations: AA, arachidonic acid; PAF, platelet activating factor; TxA_2, thromboxane A_2; ANA, *N*-arachidonoyl-ethanolamine; PMSF, phenylmethylsulfonyl fluoride

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Elbeuf, France) weighing 3–4 kg were bled from the central artery of the ear on 5 mM EDTA. Platelets were washed as already reported [35], and were resuspended at 4×10^8 cells/ml in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.6 mM glucose, 0.25% gelatin, pH 7.4). When specified, platelets were incubated 15 min with 100 μM aspirin and then washed.

2.3. Aggregation of washed platelets

Aggregation was monitored by light transmission in a PICA® (Chrono-log corporation) as described [36]. Washed platelets were incubated for 2 min with 2 mM $CaCl_2$ plus 0.28 mg/ml fibrinogen (except when thrombin was used), then stimulated as detailed in the legends of illustrations, and aggregation followed for 3 min. PAF and collagen were used at concentrations of 40 nM and 10 μg/ml, respectively.

2.4. Labelling of platelets and measurements of AA release

As already described [35], aspirin-treated platelets were incubated for 90 min at 37°C with 0.2 μCi/ml of [^{3}H]AA (from Amersham, Les Ulis, France), then washed twice to remove the radioactivity that was not incorporated. The labelled platelets were in Tyrode's buffer (pH 7.4) and completed with 2 mM $CaCl_2$ and 0.28 mg/ml fibrinogen. Aliquots (0.5 ml) of labelled platelets were incubated for 3 or 10 min at 37°C with 10 μM ANA in the absence or presence of 20 μM PMSF, with 0.25 U/ml thrombin or with saline (control). The reactions were stopped with 0.5 ml Tyrode's solution (pH 7.4) containing 5 mM EDTA and 10 mg/ml bovine serum albumin pre-cooled at 4°C. Platelets were then removed by a 2 min centrifugation at $7800\times g$, and the radioactivity, in 100 μl aliquots of the supernatant, was determined by liquid scintillation spectrophotometry.

2.5. Cleavage of exogenously added [^{3}H]ANA into [^{3}H]AA by blood platelets

[^{3}H]ANA ([arachidonyl 5,6,8,9,11,12,14,15-^{3}H]ethanolamine from NEN) at a specific activity of 221 Ci/mmol was used as tracer. A mixture of [^{3}H]ANA:ANA (ratio 1:2000) was prepared at 1 mM with a radioactivity of 0.1 mCi/ml. Then, 10 μM of [^{3}H]ANA:ANA (1:2000) was added to aspirin-pretreated platelets in the presence of 2 mM $CaCl_2$ and fibrinogen, with or without addition of PMSF. After 3 or 10 min, 200 μl of the platelet suspension was collected. AA and ANA were extracted twice by the method of Bligh and Dyer [37] and the chloroform phases were pooled and applied to thin layer chromatography plates (silica gel 60). The chromatography was performed with hexane:ethyl acetate:H_2O:acetic acid (50:100:100:20, v/v) in order to separate ANA and AA adequately [22]. After detection by iodine vapor, the spots corresponding to ANA and AA were scraped off, and the radioactivity was determined by liquid scintillation spectrophotometry.

3. Results

3.1. Action of AA and ANA on platelet aggregation

As expected, AA induced platelet aggregation in a concentration-dependent manner (Fig. 1) with an optimum at concentrations from 3 to 10 μM. At concentrations above 20 μM, a progressive decrease in the aggregating activity of AA was observed. Similar effects were observed with ANA, the concentration–response curve of which was superimposed on that of AA (Fig. 1). The loss of aggregating effect at high concentrations of AA has been explained by the production of inhibitory prostaglandins such as PGE_2 [27–31]. The fact that this phenomenon was also observed with ANA suggests the production of PGE_2 from ANA occurred in blood platelets.

The presence of calcium is necessary for agonist-induced platelet aggregation [27,28], since it allows the formation of interplatelet bridges. The fibrinogen also promotes the platelet aggregation and is necessary in vivo and in vitro to observe a proaggregant effect by AA, collagen and PAF [27,28]. We showed here that the aggregation of washed platelets by

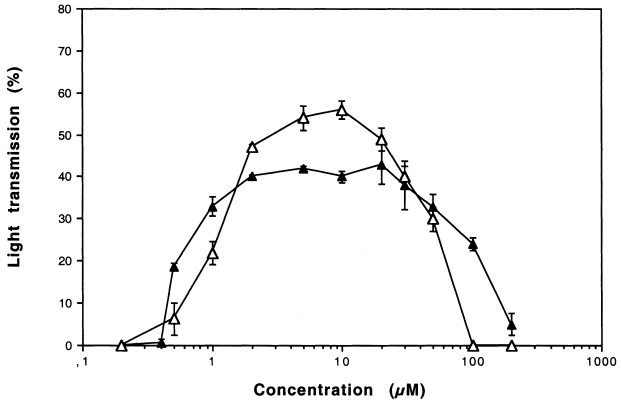

Fig. 1. Platelet aggregation induced by AA (▲) and ANA (△). Washed platelets were incubated for 2 min with 2 mM $CaCl_2$ and 0.28 mg/ml fibrinogen. AA and ANA were then added at the indicated concentrations. Values are the means ± S.E.M. of three experiments.

ANA was dependent on the presence of calcium, since only a shape change was observed in its absence (Fig. 2A). In the absence of fibrinogen, the aggregation was still observed but to a lesser extent compared to that obtained in its presence (data not shown).

Addition of AA to washed platelets, in the presence of calcium and fibrinogen, induced an immediate platelet aggregation (Fig. 2B). In contrast, platelet aggregation induced by an optimal concentration of ANA (10 μM) was initiated after a lag time of 45–60 s (Fig. 2C). This delay might reflect the occurrence of an additional step necessary for ANA-induced platelet activation.

We then examined whether this lag time was dependent on the presence of calcium. Blood platelets incubated with fibrinogen in the absence of calcium received 10 μM of ANA, then calcium was introduced at different time intervals. In the absence of calcium, only a shape change occurring after 45–60 s was observed, as shown above. When calcium was introduced 2 and 4 min after the addition of ANA, the aggregation started immediately (Fig. 2E,F). This result indicated that the extracellular calcium was only required for the aggregating effect of ANA, but not for the step preceding this aggregation.

3.2. ANA-induced aggregation was abolished by aspirin and PMSF pretreatment of blood platelets

Aspirin is known to inactivate cyclooxygenase irreversibly in various cell types. This leads to the complete inhibition of AA-induced platelet activation. Aspirin pretreatment of blood platelets was performed in order to determine whether ANA-induced aggregation was mediated by a similar pathway to AA. Results in Table 1 show that ANA-induced shape change and aggregation were completely blocked by aspirin. The addition of compound SQ-29548, a well-known TxA_2 receptor antagonist [38], completely abolished the aggregation induced by ANA (53 ± 4% and 0% of light transmission in the absence and in the presence of 1 μM SQ-29548, respectively, $n=3$). This suggests that ANA-induced platelet activation occurs through a TxA_2 receptor-dependent pathway, as described for AA.

The amidase activity described in the brain to be involved in the catabolism of ANA belongs to the PMSF-sensitive serine protease family [22–25]. It was thus interesting to ex-

Table 1
Effect of aspirin and PMSF on thrombin, ANA- and AA-induced platelet aggregation

	Thrombin (0.25 U/ml)	ANA (10 μM)	AA (20 μM)
Control (saline)	46.6 ± 0.4	56.0 ± 2.1	40.0 ± 1.5
Aspirin (30 μg/ml)	59.0 ± 3.8	0 ± 0	0 ± 0
PMSF (20 μM)	47.0 ± 0.8	0 ± 0	44.0 ± 5.1

Optimal concentrations of ANA and AA were used to determine their effect on platelet aggregation in the presence or absence of aspirin, and in the presence or absence of PMSF as compared to that of thrombin. Values are the mean ± S.E.M. of three separate experiments.

amine the effect of PMSF on platelet aggregation induced by ANA. We observed that the addition of 20 μM PMSF completely abolished the aggregating effect of ANA, as well as the accompanying shape change. In contrast, PMSF was unable to affect the aggregation induced by thrombin and AA (Table 1), as well as collagen and PAF (data not shown). Since the inhibitory effect of PMSF was specific for ANA-induced aggregation, it is likely that PMSF interferes with an amidase activity involved in the cleavage of ANA into AA.

3.3. *[^{3}H]ANA cleavage into [^{3}H]AA correlated with platelet activation induced by ANA*

To further investigate whether platelets possess a PMSF-sensitive amidase activity producing AA from exogenous ANA, [^{3}H]ANA (labelled on the AA moiety) was added to platelets, and the appearance of [^{3}H]AA was examined. Our results showed that [^{3}H]ANA was time-dependently converted into a radioactive compound which comigrated with AA on thin layer chromatography (Fig. 3A), and was concomitant with a decrease in [^{3}H]ANA content (data not shown). The half-life of [^{3}H]ANA in platelets was around 10 min in our experimental conditions (Fig. 3A). The cleavage of ANA was completely abolished when PMSF was added to platelets before the addition of [^{3}H]ANA. Thus, we concluded that platelets possess a specific PMSF-sensitive amidase activity converting ANA into AA, the latter being involved in platelet activation.

Finally, a possible effect of ANA via binding on a receptor that might elicit liberation of endogenous AA, and consequently platelet aggregation, was examined. We first investigated whether the addition of ANA on [^{3}H]AA-labelled platelets, pretreated with aspirin, was able to release [^{3}H]AA from platelets. Aspirin was used in this experiment to block the metabolism of [^{3}H]AA and to avoid the production of AA derivatives that could activate platelets and induce further AA liberation. Our results showed that ANA failed to induce the liberation of [^{3}H]AA from platelets (Fig. 3B). Compound HU-210, a CB1 and CB2 cannabinoid receptor agonist [39], failed to induce platelet activation even at concentrations up to 20 μM (Fig. 3C). Compound SR-141716, a CB1 receptor antagonist [40], was unable to block the aggregating effect induced by ANA (Fig. 3C). These findings suggest that ANA-induced aggregation does not occur via a cannabinoid receptor-dependent pathway.

4. Discussion

In this study, we report that micromolar concentrations of ANA stimulate platelets, leading to a shape change and aggregation. We also show that AA and ANA concentration–response curves are superimposed and that their activating

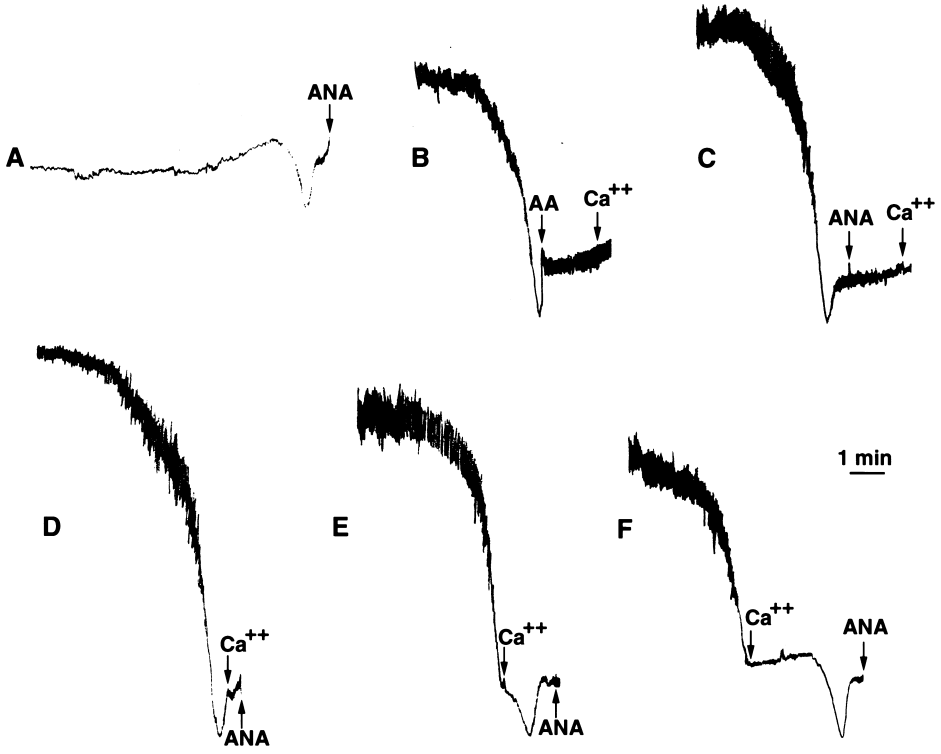

Fig. 2. Kinetic of ANA-induced platelet aggregation. Washed platelets were incubated for 2 min with 0.28 mg/ml fibrinogen, and ANA was added at the optimal concentration (10 μM). Then 2 mM $CaCl_2$ was added at the indicated time. Representative recordings of platelet aggregation are shown.

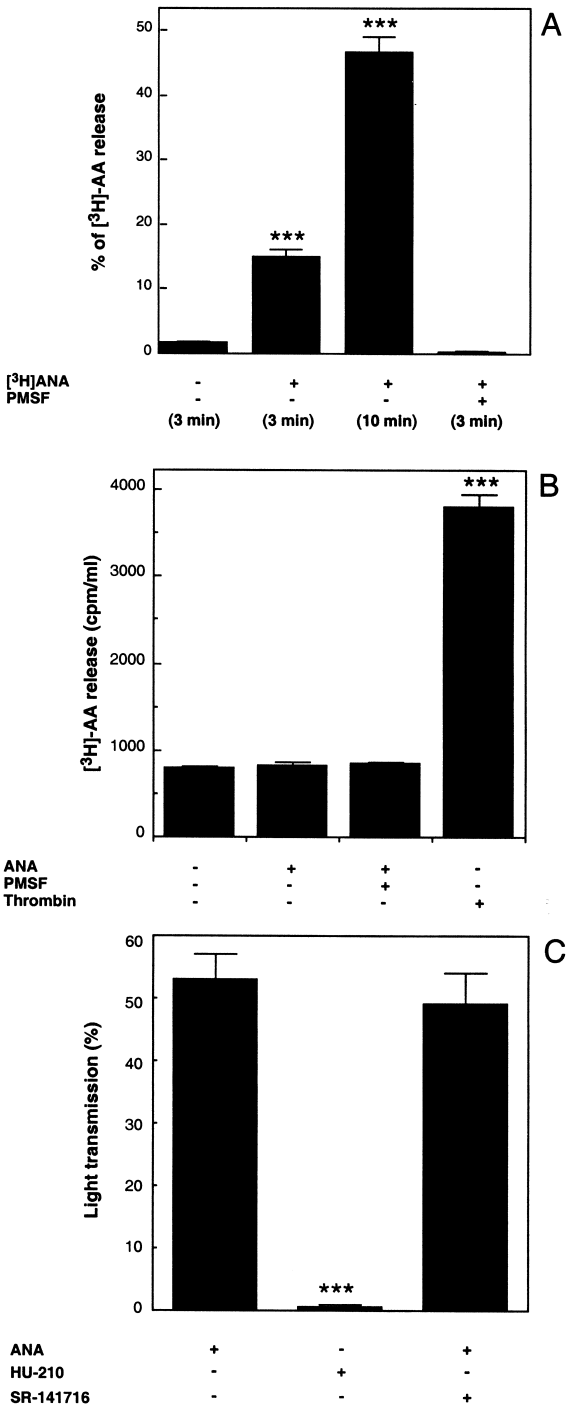

Fig. 3. Effect of ANA on [^{3}H]AA release and effect of compounds HU-210 and SR-141716 on ANA-induced platelet aggregation. A: The appearance of [^{3}H]AA was measured after addition of 10 μM [^{3}H]ANA on aspirin-treated platelets in the presence or absence of 20 μM PMSF. B: The liberation of [^{3}H]AA from platelet membrane was measured after addition of 10 μM ANA on aspirin-treated platelets labelled with [^{3}H]AA in the absence or presence of 20 μM PMSF. C: The effect on blood platelets of 4 μM ANA, 20 μM HU-210, and 4 μM ANA in the presence of 4 μM SR-141716 was measured in the absence of aspirin. Values are the means ± S.E.M. of three experiments.

effects are abolished by pretreatment of platelets with aspirin. This leads to the conclusion that cyclooxygenase is involved in the action of ANA and it indicates that AA and ANA induce platelet activation by similar mechanisms.

When added on blood platelets, AA immediately induces platelet shape change and aggregation, while a lag time of almost 1 min was observed with ANA (Fig. 2). This delay may represent the time required for the cleavage of ANA into AA. Indeed, a specific amidase, sensitive to PMSF and calcium-independent, has been described in brain able to cleave ANA [22–25]. A low concentration of PMSF specifically abolished the activating effect of ANA on platelets. Finally, [^{3}H]ANA was time-dependently converted into [^{3}H]AA through a process blocked by PMSF. Altogether, our observations support the hypothesis that ANA is cleaved by a PMSF-sensitive amidase in platelets, and that this cleavage correlates with the ANA-induced platelet activation. The appearance of AA will lead to the formation of AA derivatives, such as TxA_2 which is a potent platelet activator through binding to a specific receptor. In agreement, compound SQ-29548, a well-known TxA_2 receptor antagonist, completely abolished the activating effect of ANA, further supporting the conclusion that ANA-induced platelet activation occurs via an AA-dependent pathway.

It has been shown that ANA exerts various peripheral effects through binding to either CB1 or CB2 receptors [9,10]. Binding of ANA or cannabinoids on a receptor expressed in fibroblasts increased AA release via the stimulation of the mitogen-activated protein kinase signalling pathway [41]. In order to examine whether ANA induces AA release via a receptor-dependent mechanism, platelets were pretreated with aspirin and labelled with [^{3}H]AA, and then stimulated with ANA. We did not observe any [^{3}H]AA liberation indicating that the activating effect of ANA is not due to the release of endogenous AA from the platelet membrane via ANA binding to a receptor. In agreement, a highly potent cannabinoid receptor agonist (compound HU-210) failed to activate blood platelets and a cannabinoid receptor antagonist (compound SR-141716) was unable to block ANA-induced platelet aggregation. These results provide further reasons to discard the hypothesis that the amidase activity might be activated after ANA binding on a receptor.

It is obvious from our study that the platelet activating effect of ANA is based on its cleavage into AA. However, we cannot exclude that a part of exogenous ANA might be directly metabolized by either cyclooxygenase or 12-lipoxygenase in platelets as reported [32–34], especially because we did not observe a total cleavage of ANA. Recently, ANA has been shown to be metabolized to a limited extent by pre-activated human platelets into 12(*S*)-HAEA [42], but no data are available on ANA metabolization in resting platelets. However, since the activating effect of ANA is completely abolished by PMSF at a concentration which does not interfere with the effect of AA, it is likely that the main action of PMSF is to inhibit the amidase activity that catalyzes the cleavage of ANA into AA.

In conclusion, ANA is a potent aggregating agent in rabbit platelets via its cleavage into AA. However, the physiological and/or pathophysiological significance of platelet activation by ANA has still to be examined. Together with recent studies showing the involvement of ANA in inflammatory, immune or cardiovascular systems, our results support the hypothesis

that ANA may play important roles in functions other than those described in the nervous system.

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