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LETTER TO THE EDITOR

# Activation of platelets by the endocannabinoids 2-arachidonoylglycerol and virodhamine is mediated by their conversion to arachidonic acid and thromboxane A2, not by activation of cannabinoid receptors

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**History**

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**To the editor,**

In his corresponding letter to the editor, Dr. Maccarrone addresses our recent article in the Journal which ends with his belief *''that platelet activation by eCBs (endocannabinoids) still remains an open and rather complex question''* [1]. Based on the results of our study which are fairly straight-forward and merge into a simple conclusion we do not share his opinion [2]. Our conclusions concerning the platelet activation mechanism induced by 2-arachidonoylglycerol (2-AG) in blood and platelet-rich plasma (PRP) are also in agreement with a previous study [3].

We would like to clarify and stress two main points:

(1) We found that the two endocannabinoids 2-AG and virodhamine stimulate platelets in blood and platelet-rich plasma, whereas the endocannabinoid anandamide (AEA) and the synthetic cannabinoid arachidonyl 20-chloroethylamide (ACEA) did not. The chemical structure of these cannabinoids shows that arachidonic acid in 2-AG and virodhamine is linked to the rest of the molecule via an ester linkage, whereas in AEA and ACEA arachidonic acid has an amide linkage (see also Table 1 of Ref. [2]). 2-AG and virodhamine are preferred substrates for monoacylglycerol lipase (MAGL) which cleave the ester bond of 2-AG and virodhamine, whereas AEA and ACEA are substrates for fatty acid amidohydrolase (FAAH), which hydrolyzes the amide bond of AEA and ACEA [4–7]. Although 2-AG can also be cleaved by FAAH *in vitro* [8], MAGL is the main degrading enzyme *in vivo* [5, 7].

For the understanding of our results, it is important to consider the respective localization of MAGL and FAAH. MAGL is localized not only intracellularly (as stated in the letter of Dr. Maccarrone), but also extracellularly. MAGL, also known as monoglyceride lipase or monoglyceride hydrolase [9], is part of the enzymatic machinery which degrades lipoprotein triglycerides, and is present in plasma [10, 11]. Moreover intact platelets exhibit MAGL activity [11], and MAGL has been found in platelet membranes [4] supporting the idea of MAGL expression at the platelet plasma membrane surface.

The extracellular localization of MAGL is apparently in contrast to FAAH, for which an extracellular localization has not been reported [8]. The different localization of MAGL and FAAH is the most simple explanation of our results of platelet activation in blood and PRP by 2-AG and virodhamine (substrates of MAGL), but not for anandamide and arachidonyl 20-chloroethylamide (ACEA) (substrates of intracellular FAAH).

Careful reading of our article shows that all this has more or less been written in our article [2]; see Discussion on p. 8:

*''MAGL is present in plasma and platelets, and MAGL as opposed to FAAH is the main enzyme responsible for 2-AG hydrolysis in cells including human platelets [30, 44, 45]''. and: ''The lack of platelet activity of anandamide also indicates that anandamide in blood or PRP does not reach intracellular FAAH in platelets [30] and other blood cells [42].''*

Moreover, we found that arachidonic acid activated platelets in blood in the same concentration range as 2-AG (see Figure 6 of Ref. [2]). Thus, a platelet endocannabinoid transporter as emphasized by Dr. Maccarrone in his letter is not needed to explain our results.

(2) In his letter, Dr. Maccarrone expressed concern about the apparently high concentrations of some of the inhibitors used. *''The conclusions of Brantl and colleagues were essentially based on the ability of admittedly high doses of acetylsalicylic acid (5.4 mM), a well-known cyclooxygenase inhibitor [7], and of JZL184 (20 μM), a selective inhibitor of MAGL at nanomolar concentrations (IC50 ~8 nM) [8], to block the effect of 2-AG [6]''*. However, careful reading of our article shows not only an explanation for the concentrations of JZL184 and ASA used but also that lower concentrations of these inhibitors provided the same results.

(a) We explained the concentrations of JZL and ASA used in our article:

- Concerning JZL184 see p. 3 of our article: *''A concentration of 20 μM of JZL184 was chosen, since it has been reported that this concentration did not show unspecific effects on other*

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*lipid enzymes (diacylglycerol lipase, cytosolic phospholipase A2) recombinantly expressed in HEK293 cells [36]. The concentrations of JZL184, which inhibit MAGL in human plasma or blood, have not been reported.''*

The IC50 of 8 nM JZL184 was found in a MAGL enzyme assay using mouse membrane fractions [5]. The higher concentrations of JZL184 required for inhibition of MAGL in blood and PRP can be explained by the binding of this amphiphilic substance to plasma proteins and cell membranes. We would like to mention here that we observed for JZL184 also a time-dependent inhibition of 2-AG- and virodhamine- induced platelet activation (data not shown), which supports the covalent mechanism of inactivation of MAGL reported for this inhibitor (Supplementary Figure 2 online of Ref. [5]) and the specificity of this inhibitor in our experiments.

- Concerning the use of acetylsalicylic acid (ASA) see p. 3 of our article: *''ASA was used at 5.4 mM due to the short pre-incubation time (3 min) in blood. We have shown previously that ASA at 5.4 mM incubated for 3 min with blood was equipotent to ASA 1mM incubated for 30 min with blood, and that both concentrations inhibit specifically platelet aggregation induced by AA, but not ADP [37].''*

Therapeutic doses of ASA result in plasma levels of the drug in the range between 0.1 and 0.5 mM, and 1 mM ASA incubation for 10–15 min at 37 °C is commonly used to block completely platelet cyclooxygenase–dependent platelet aggregation in PRP and blood [12–14].

(b) We showed that lower concentrations of these inhibitors produced the same results (see p. 4 of our article):

- *''We also studied shape change under conditions, where this early platelet response is induced selectively without aggregation and secretion (Figure 5). Under these conditions platelet shape change is independent of positive feedback mediators such as released ADP or TXA2 and reflects direct platelet activation by the added stimulus [38]. Furthermore, we applied lower concentrations of JZL184 (10 μM), ASA (1 mM) and daltroban (20 μM) to minimize possible unspecific effects of these inhibitors. We found that shape change induced by 2-AG and virodhamine under these conditions was also completely inhibited by these three inhibitors (Table III).''*

(c) We also used a specific antagonist of the TXA2 receptor (daltroban).

We obtained the same results with daltroban as with ASA and JZL184. This is important and has not been mentioned in the letter of Dr. Maccarrone. Our results with daltroban show that platelet activation induced by virhodamine and 2-AG is mediated by stimulation of $TXA_2$ receptors. These results do not leave any room for speculations made in the letter that other oxygenated arachidonate derivatives with distinct biological activities may be involved [1].

Finally, the results concerning the observations of 2-AG in blood and PRP made by us are in complete agreement with the previous study of Keown et al. (see also Discussion of our article). These authors found inhibition of 2-AG- induced platelet aggregation in blood by ASA not only *in vitro* and but also *ex vivo* (after ingestion of the drug by patients), and *in vitro* in blood and PRP with a MAGL inhibitor (URB602) different than applied in our study [3]. In addition, in our study synthetic CB1 and CB2 receptor agonists completely lacked any effect on platelet activation or inhibition, and in the study of Keown et al. CB1 and CB2 receptor antagonists did not inhibit 2-AG –induced platelet activation.

In conclusion, the experimental evidence clearly indicates that platelet activation induced by the endocannabinoids 2-AG and virhodamine in blood and platelet-rich plasma proceeds through a mechanism which involves MAGL-induced endocannabinoid hydrolysis and the arachidonate/thromboxane A2 pathway, but not G-protein coupled CB1/CB2 receptors, or a non-CB1/CB2 type receptor [2, 3].

## Declaration of interest

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