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Blockade of ADP-induced Ca^{2+}-signal and platelet aggregation by lipoxygenase inhibitors

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Stimulation of platelets results in the liberation of arachidonic acid (AA) which is further metabolized via the cyclooxygenase or lipoxygenase (LPG) pathway. We have examined the effect of inhibition of LPG on (i) the ADP-induced increase of cytoplasmic Ca^{2+} concentration and (ii) platelet aggregation. Lipoxygenase inhibitors, nordigidroguaiaretic acid (NDGA) and BW-755C, both suppressed ADP-induced Ca^{2+}-signals and aggregation in a dose-dependent manner, with an IC_{50} value of 1–2 μM for NDGA. Qualitatively the same effect was obtained with 4-bromophenylacyl bromide, the inhibitor of phospholipases A_2 and C. By contrast, cyclooxygenase inhibitor indomethacin had only a negligible effect on Ca^{2+}- signals and suppressed only the second phase of ADP-induced aggregation. It is concluded that the LPG pathway of AA metabolism in platelets might play a crucial role in ADP-induced Ca^{2+}-signal generation and platelet aggregation.

Ca^{2+}-signal; Platelet aggregation; Lipoxygenase inhibitor

1. INTRODUCTION

In platelets as well as in other cells, AA is metabolized through both the cyclooxygenase and lipoxygenase pathway (for review see [1]). The involvement of cyclooxygenase AA metabolites in the regulation of platelet functions is well established [2–4]. Thus it has been shown that the cyclooxygenase blockade by indomethacin suppresses the second phase of platelet aggregation and dense granule secretion caused by ADP and other 'weak agonists' [5,6]. Indomethacin, however, does not diminish the stimulus-induced increase in cytoplasmic Ca^{2+} concentration (Ca^{2+} signal) [7]. Therefore, the conclusion was drawn that thromboxane A_2 (TXA_2), a potent aggregatory agent, synthesized from AA via the cyclooxygenase pathway [1] is not a prerequisite for ADP-induced Ca^{2+}-signal generation [7].

The data concerning the role of the lipooxygenase pathway during AA metabolism in the mechanism of platelet activation are at present scant and controversial. Thus, it has been found that the application of the LPG inhibitor, NDGA, caused partial inhibition of ADP-induced platelet aggregation [8]. However, 12-HETE, one of the LPG metabolites of AA, upon external application exerted an inhibitory influence on AA-induced platelet aggregation [9].

To date, the information concerning the role of the LPG pathway in mechanisms of platelet activation has mainly been obtained from aggregation studies. Only one study has demonstrated the partial inhibitory effect of NDGA on the Ca^{2+} signal induced in human platelets by thrombin. We failed to find any indication to the effect of LPG inhibitors on ADP-induced Ca^{2+} signals in platelets. On other cell types, e.g., rat thymocytes, Gukovskaya et al. [12] observed that the blockade of LPG with NDGA suppresses the concanavalin A-induced Ca^{2+} signal.

The object of the present study was to examine the contribution of the LPG pathway during AA metabolism to the mechanism of Ca^{2+}-signal generation and platelet aggregation induced by ADP. As tools to study this problem, we have used two LPG inhibitors, NDGA and BW-755C [11,12], as well as 4-bromophenylacyl bromide, an irreversible inhibitor of phospholipases A_2 and C [11,13]. The effects observed were compared with the action of the cyclooxygenase inhibitor indomethacin [11].

The results of the present study show that in human platelets a blockade of the LPG pathway during AA metabolism can cause an effective concentration-

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Abbreviations: AA, arachidonic acid; LPG, lipoxygenase; TXA_2, thromboxane A_2; NDGA, nordihydroguaiaretic acid; 12-HETE, 12-*s*-hydroxy-5,8-*cis*-10′-*trans*-14-*cis*-eicosotetraenoic acid; 12-HPETE, 12-*s*-hydroperoxy-5,8-*cis*-10′-*trans*-14-*cis*-eicosotetraenoic acid; ACD, acid-citrate-dextrose anticoagulant

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dependent inhibition of both Ca^{2+}-signal generation and platelet aggregation caused by ADP.

2. EXPERIMENTAL

Freshly drawn blood taken with ACD anticoagulant (6:1) was centrifuged at 200 × g for 15 min to obtain platelet-rich plasma (PRP). The PRP was then incubated for 20 min with 20 μM Quin-2 acetoxymethyl ether (Sigma) at 37°C. PRP was centrifuged after the addition of 50 μl/ml ACD anticoagulant at 350 × g for 10 min. The pellet was resuspended in modified Tyrode's buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 10 mM Hepes, pH 6.8) to a final concentration of 3–4 × 10^8 cell/ml. Apyrase (20 μg/ml) was added to prevent activation by residual traces of ADP. Prior to Ca^{2+}-signal measurements 100 μl of Quin-loaded platelets were diluted to 1 ml with buffer (composition described above), pH 7.4, and 1 mM of $CaCl_2$ was added. The Quin-2 fluorescence was measured in a Hitachi 4000 spectrofluorimeter, the experimental details for the calibration of Quin-2 signals being identical to those described in the literature [14].

Platelet aggregation was measured in a Chronolog (USA) lumiaggregometer at 37°C and at a stirring speed of 900 rpm. Platelets washed from PRP and resuspended as described above in buffer, pH 7.4 (37°C), to a final concentration of 3–4 × 10^8 cell/ml. 0.5 ml of suspension was placed in an aggregometer cuvette, subsequently 1 mM $CaCl_2$ and 0.8 mg/ml fibrinogen (Serva) were added.

In experiments with inhibitors, the cells were incubated, 5 min prior to ADP (10 μM final, Sigma) activation, with either correspondent inhibitor or vehicle (control).

3. RESULTS AND DISCUSSION

In our experiments, 10 μM ADP produced a rise in $[Ca^{2+}]_i$ from the basal level of 120 ± 10 nM to 700 ± 80 nM (n = 20). Fig.1A shows typical ADP-induced Ca^{2+} signals in cells suspended in control medium (curve 1) or in medium containing 20 μM NDGA. In the latter case, Ca^{2+} signal was almost completely abolished. The dose-response curve for the effect of NDGA on ADP-induced Ca^{2+} signals (fig.1B) gives an IC_{50} value of about 1 μM. A similar dose-response relation was obtained in studying the effect of NDGA on platelet aggregation (fig.2).

The IC_{50} value proved to be close to those obtained for NDGA-induced LPG pathway inhibition on neurones and neutrophils [15,16]. Another lipooxygenase inhibitor, compound BW-755C, also caused an

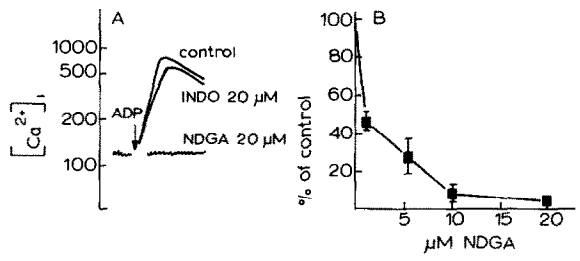

Fig.1. (A) Effect of NDGA and indomethacin on ADP-induced Ca^{2+} signals in platelets. Representative fluorescent tracing from 3–8 experiments are shown. ADP, 10 μM. (B) Dose-response dependence of NDGA inhibitory effect. 100% – $[Ca^{2+}]_i$ increase in control sample.

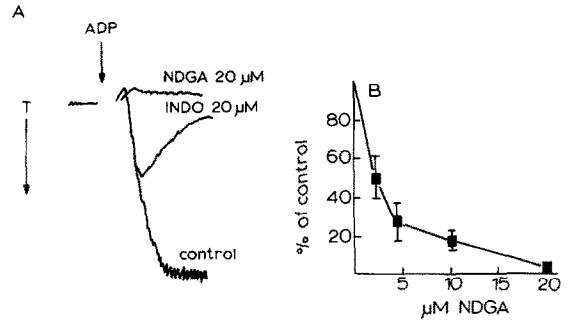

Fig.2. (A) Effect of NDGA and indomethacin on ADP-induced platelet aggregation. Representative aggregation tracing from 3–5 experiments are shown. ADP, 10 μM. (B) Dose response dependence of NDGA inhibitory effect on platelet aggregation. 100% – aggregation amplitude in control sample.

effective inhibition of platelet aggregation and Ca^{2+} signal (fig.3).

In order to ascertain that the effects of NDGA and BW-755C described resulted from altered AA metabolism, we performed experiments with platelets treated with 4-bromophenylacyl bromide, the inhibitor of phospholipase A_2 and C, which is known to prevent AA liberation from membrane phospholipids [11,13].

Fig.3 shows that addition of 20 μM 4-bromophenylacyl bromide to a platelet suspension led to a highly significant inhibition of both Ca^{2+} signal and platelet aggregation.

In contrast to the LPG and phospholipase inhibitors,

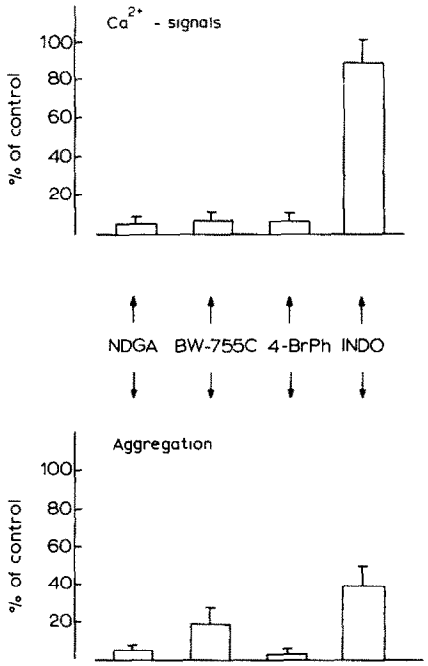

Fig.3. Effect of inhibitors of AA metabolism on ADP-induced Ca^{2+} signals (upper panel) and platelet aggregation (lower panel). All inhibitors were used at a concentration of 20 μM. 100% – platelet response in control cells.

the inhibitor of cyclooxygenase, indomethacin (20 μM), caused only a very small decrease in Ca^{2+} signal (see fig.1) and a partial suppression of platelet aggregation, which at the same time became partially reversible (fig.2). The Ca^{2+}-signal resistance in ADP-activated platelets to indomethacin has been reported earlier [7]. A small decrease in this signal shown in fig.1 may be attributed to side effects of indomethacin, since at a concentration higher than 5 μM it also affects the LPG [11].

These experiments suggest a crucial role for the LPG pathway in the generation of Ca^{2+} signals and aggregation of human platelets for which there are two possible explanations.

On the one hand, one or more LPG metabolites of AA could leave the stimulated cell, in which they are generated, and act as first messengers on the platelet membrane receptors, thus activating in some unknown fashion receptor-operated Ca^{2+} channels. This suggestion, however, is not supported by the following data. As mentioned above, 12-HETE, one of the principal LPG metabolites of AA in human platelets [1], exerts an inhibitory influence on platelet aggregation caused by AA, when applied externally [9]. However, the effect of 12-HETE on ADP-induced platelet aggregation has not yet been studied. According to Westlund et al. [17] LPG metabolism of AA is not terminated by the generation of 12-HETE and 12-HPETE in platelets, but leads to formation of a leukotriene-like substance and dihydroxyeicosanoid acids. Leukotrienes in other cell types (e.g. lymphocytes, neutrophils) are known to induce activation of Ca^{2+} influx in response to stimulation [18]. Thus, it is reasonable to search for LPG metabolites of AA in platelets that could be involved in their autocoid activation.

On the other hand, some of the LPG metabolites of AA may play a role as intracellular second messengers whose generation is a prerequisite for ADP-induced activation of Ca^{2+} channels. Here one could speculate about a possible effect of this metabolite on G-proteins mediating the receptor Ca^{2+}-channel coupling.

Whatever the exact mechanism of LPG products involved in Ca^{2+}-signal regulation, maybe their participation is important for understanding the mechanism of platelet activation.

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