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Lack of evidence for a role for the lipoxygenase pathway in increases in cytosolic calcium evoked by ADP and arachidonic acid in human platelets

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We have investigated the possibility that metabolism of arachidonic acid by the lipoxygenase pathway contributes to ADP-evoked rises in $[Ca^{2+}]_i$ in human platelets. 30μM BW A4C did not affect ADP-evoked Ca^{2+} signals, but inhibited 12-lipoxygenase activity in platelet homogenates. Another lipoxygenase inhibitor, MK 866 was similary without effect on ADP-evoked Ca^{2+} signals. ADP was found to liberate little arachidonic acid, and formation of the lipoxygenase product 12-HETE was not detectable. The rise in $[Ca^{2+}]_i$ evoked by arachidonic acid was completely inhibited by the cyclooxygenase inhibitors aspirin or indomethacin. These results indicate that lipoxygenase products do not play an essential role in mediating rises in $[Ca^{2+}]_i$ evoked by ADP, or by arachidonic acid.

ADP; Arachidonic acid; Calcium; Fluorescent indicator; Lipoxygenase inhibitor; Platelet

1. INTRODUCTION

Platelet activation by various agonists is accompanied by the liberation of arachidonic acid which is subsequently metabolised by cycloxygenase and lipoxygenase pathways [1]. One of the main cycloxygenase products, TxA_2, is recognised as having an important autocoid role, itself stimulating the hydrolysis of phosphatidylinositol 4,5-bisphosphate and elevating $[Ca^{2+}]_i$ [2,3]. These actions reinforce the primary stimulus, but, with the exception of activation by collagen, are not essential for an elevation in $[Ca^{2+}]_i$, which can occur when cycloxygenase is inhibited using aspirin or indomethacin [4,5].

Recently, it has been suggested that the metabolism of arachidonic acid by the lipoxygenase pathway plays a crucial role in ADP-evoked Ca^{2+}-signal generation and aggregation in human platelets [6]. This proposal was based on the finding that NDGA and BW 755C, which have lipoxygenase-inhibiting activity, suppressed ADP-evoked activation. However, the specificity of these compounds is relatively poor, for example BW 755C has similar inhibitory effects on lipoxygenase and cycloxygenase activity [7]. The proposal that lipoxygenase products play a crucial intermediary role in the ADP response contrasts with evidence that this agonist can elevate $[Ca^{2+}]_i$ without measurable delay (≤30 ms) in human platelets [8.9], apparently by opening receptor-operated non-selective cation channels in the plasma membrane [10].

Here we report a re-examination of the role of the lipoxygenase pathway in the generation of Ca^{2+} signals evoked by ADP and arachidonic acid in human platelets co-loaded with fura-2 and BAPTA. We have compared the effects of NDGA with those of the more specific lipoxygenase inhibitors BW A4C [7] and MK 866 [8] on basal $[Ca^{2+}]_i$ and on ADP-evoked changes. A contribution of the lipoxygenase pathway to Ca^{2+} signal generation following the metabolism of arachidonic acid was also tested for by comparing responses before and after cycloxygenase inhibition using aspirin or indomethacin.

2. EXPERIMENTAL

Platelet-rich plasma was prepared as previously described [4]. After the addition of apyrase (20 μg/ml) and, if appropriate, aspirin (100 μM), the cells were loaded with fura-2 and BAPTA by incubation with fura-2/AM (5 μM) and BAPTA/AM (10 μM) for 45 min at 37°C. (Cells were co-loaded with BAPTA to increase cytosolic Ca^{2+} buffering and make any inhibition of Ca^{2+} signal generation easier to detect.) Platelets were then collected by centrifugation and resuspended in HEPES-buffered saline of composition (in mM): 145 NaCl, 5 KCl, 1 $MgSO_4$, 10 Na-HEPES, pH 7.4, at 37°C. When investigating the effects of cycloxygenase inhibition, the previously untreated cell suspension was divided and half was treated with aspirin (100 μM) or

Abbreviations: BW A4C, *N*-(4-benzyloxybenzyl)-acetohydroxamic acid; MK 866, (3[3-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]2,2-dimethyl-proanoic acid; $[Ca^{2+}]_i$, cytosolic calcium concentration; NDGA, nordihydroguaiaretic acid; 12-HETE, 12-*s*-hydroxy-5,8-*cis*-10'-*trans*-14-*cis*-eicosotetraenoic acid.

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indomethacin (15 μM) for 15 min before use. In other experiments, all the cells were pretreated with 100 μM aspirin to exclude any contribution of the cycloxygenase pathway to ADP-evoked responses.

Fura-2-fluorescence was recorded from aliquots of platelet suspension placed in cuvettes in a modified Perkin-Elmer MPF 44A Spectrophotometer. The suspension was thermostatically controlled at 37°C and stirred by a magnetic bar. 1 mM $CaCl_2$ was added to each aliquot before use. The fluorescence signal, with excitation at 339 nm and emission at 500 nm was calibrated in terms of $[Ca^{2+}]_i$ as previously described [5].

The conversion of [^{14}C]arachidonic acid to ^{14}C-labelled 12-HETE by platelet homogenate was determined essentially as previously described [7]. The percentage conversion to 12-HETE was calculated following quantification of activity on TLC plates using a Phosphor Imager (Molecular Design Ltd). For experiments with intact platelets, the cells were pre-labelled by incubating washed suspensions with [^{14}C]arachidonic acid (0.1 μCi/ml) for 1 h, followed by a further centrifugation and resuspension. Lipids were extracted and analysed as for homogenates.

NDGA, BW A4C and MK 866 were added from stocks in DMSO by 1000-fold dilution; aspirin and indomethacin were similarly added from stocks in methanol. The vehicles were without significant effect. Arachidonate was added from a 100 mM stock prepared by dissolving arachidonic acid in 125 mM NaOH. ADP was added from a 10 mM stock in HEPES-buffered saline.

Aspirin, arachidonic acid and indomethacin were from Sigma (Poole, UK), NDGA was obtained from Sigma and Aldrich (Gillingham, UK), Fura-2/AM and BAPTA/AM were from Molecular Probes (Eugene, OR, USA). [1-^{14}C]arachidonic acid was from Amersham International (Amersham, UK). BW A4C and MK 866 were gifts from Dr. Laurence Garland, Wellcome Research Laboratories (Beckenham, UK).

3. RESULTS AND DISCUSSION

3.1. *Effects of NDGA and 4-bromophenylacyl bromide*

NDGA from Sigma had little inhibitory effect on rises in $[Ca^{2+}]_i$ evoked by 10 μM ADP at concentrations up to 10 μM (Figs. 1a and 2a). At higher concentrations ($\geq$30 μM), the NDGA itself elevated $[Ca^{2+}]_i$, and the subsequent ADP-evoked response was reduced (Figs. 1b and 2a). In view of the activitation of the cells by this batch of NDGA, we also obtained this compound from another source (Aldrich). Again we found that higher concentrations ($\geq$30 μM) of NDGA elevated $[Ca^{2+}]_i$ (Fig. 1c), although with this batch of compound some reduction in ADP-evoked rises in $[Ca^{2+}]_i$ was observed at 10 μM and inhibition was nearly complete at 30 μM (Figs. 1c and 2b). We also attempted to use 4-bromophenylacyl bromide, an inhibitor of phospholipases A_2 and C, which Borin et al. [6] reported to inhibit responses evoked by ADP. However, we found that this compound itself induced large rises in $[Ca^{2+}]_i$ which made it impossible to assess its effects on ADP-evoked rises in $[Ca^{2+}]_i$ (not shown).

These results contrast with those of Borin et al. [6] who report almost complete inhibition of ADP-evoked rises in $[Ca^{2+}]_i$ by 10 μM NDGA and do not refer to any effects of the compound itself on $[Ca^{2+}]_i$. We cannot readily explain these differences, although they may arise from the use of quin-2-loaded platelets rather than fura-2-loaded cells, and the storage of cells at reduced pH by Borin et al. [6].

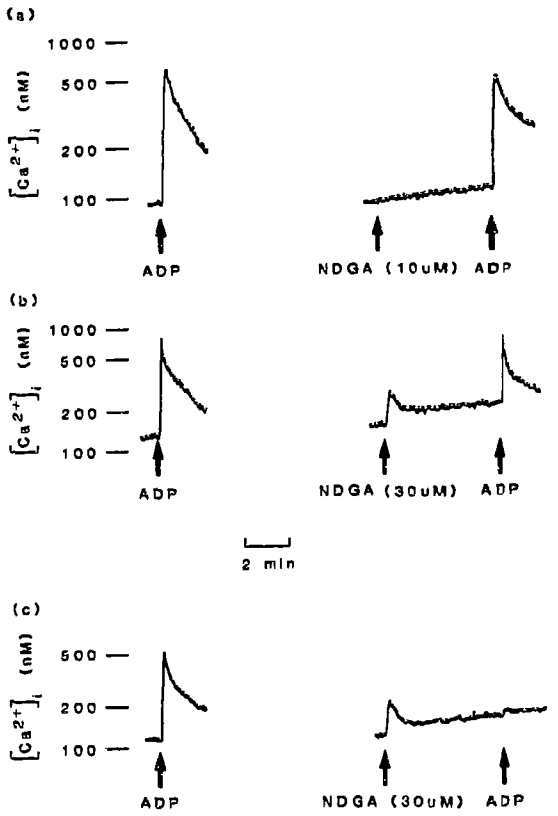

Fig. 1. Effects of NDGA on resting and ADP-evoked rises in $[Ca^{2+}]_i$. NDGA at the shown concentrations from Sigma (a,b) or Aldrich (c) and ADP (10 μM) were added as indicated by the arrows.

3.2. *Effects of selective lipoxygenase inhibitors*

In view of the difficulties in assessing the inhibitory action on the ADP response when NDGA itself activated the cells, and in the light of the known poor specificity of this compound [7], we turned to the more selective compound BW A4C. This has been reported to inhibit [^{14}C]12-HETE production from [^{14}C]arachidonic acid in platelet homogenates with an IC_{50} of 1.2 μM [7]. We also assessed the effect of the compound MK 866, which inhibits 5-lipoxygenase by blocking the translocation of the enzyme to the membrane protein FLAP with a reported IC_{50} around 1 nM [11,12]. Although platelets lack 5-lipoxygenase, the possible role of FLAP in the activation of platelet 12-lipoxygenase has yet to be determined.

BW A4C had no significant effect on the rise in $[Ca^{2+}]_i$ evoked by 10 μM ADP at concentrations up to 30 μM (Figs. 2c and 3a). This concentration of the compound reduced the conversion of [^{14}C]arachidonic acid to [^{14}C]12-HETE by platelet homogenates to about 15% of the control value, confirming its potency as an inhibitor of 12-lipoxygenase (Fig. 4). MK 866, at concentrations up to 100 nM, was without effect on the rises in $[Ca^{2+}]_i$ evoked by 10 μM ADP (Figs. 2d and 3b). These results indicate that the lipoxygenase pathway plays no essential role in the ADP-evoked rise in $[Ca^{2+}]_i$.

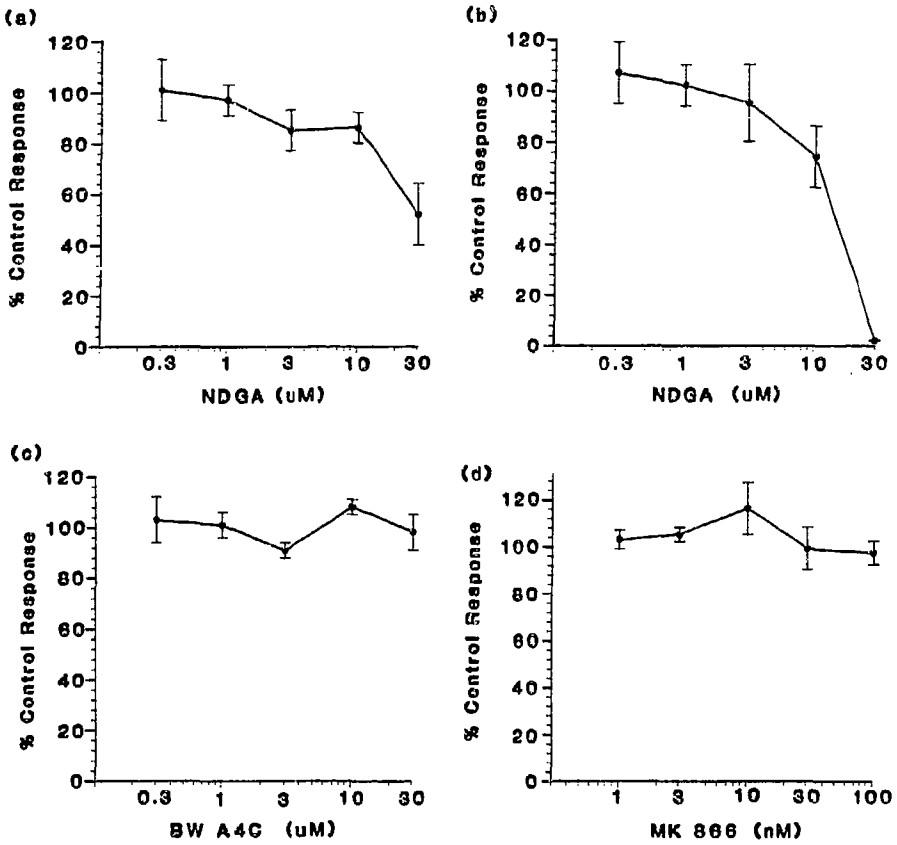

Fig. 2. Dose–response relationships for the effects on ADP-evoked rises in $[Ca^{2+}]_i$ of (a) NDGA (Sigma), (b) NDGA (Aldrich), (c) BW A4C and (d) MK 866. Results are expressed as the mean ± SE of the rise in $[Ca^{2+}]_i$ above the pre-stimulus level evoked by 10 μM ADP after 5 min pre-incubation with the inhibitor, compared with that evoked in paired controls.

3.3. *12-HETE production*

ADP is a comparatively weak platelet agonist, for example in terms of its ability to stimulate phospholipase C [13,14] or to stimulate the second phase of aggregation in the absence of thromboxane A_2 formation [15,16]. We therefore investigated the ability of ADP to evoke the production of 12-HETE in platelets pre-labelled with [^{14}C]arachidonic acid. There was no detectable production of 12-HETE in response to 10 μM ADP (not shown). This finding is in agreement with other reports that ADP evokes little or no eicosanoid formation [17], and further indicates the lack of a role for lipoxygenase in the ADP response.

3.4. *Effects of arachidonic acid*

Although our results indicate that metabolism of arachidonic acid by the lipoxygenase pathway plays no role in the generation of platelet Ca^{2+} signals evoked by ADP, this does not rule out a contribution of this pathway to the responses evoked by other agonists which may be more potent liberators of arachidonate. We therefore attempted to reveal any contribution of the lipoxygenase pathway to the rise in $[Ca^{2+}]_i$ evoked by arachidonic acid by blockade of the contribution of the cyclooxygenase pathway using aspirin or indomethacin. We also determined the effects of these inhibitors on the ADP response. The results are shown in Fig. 5.

The addition of 10 μM arachidonic acid evoked a rise in $[Ca^{2+}]_i$ of 606 ± 86 nM above the basal level, compared with a rise of only 5 ± 1 nM in paired controls pretreated with 100 nM aspirin (SE, n=8). Similarly, pretreatment with 15 μM indomethacin reduced the rise in $[Ca^{2+}]_i$ evoked by 10 μM arachidonic acid from 661 ± 48 nM to only 6 ± 1 nM in paired control (SE, n=8). In contrast, 100 μM aspirin only resulted in a reduction in the rise in $[Ca^{2+}]_i$ evoked by 10 μM ADP from 416 ± 78 nM to 364 ± 53 nM (SE, n=9). Indomethacin (15 μM) reduced the rise in $[Ca^{2+}]_i$ evoked by 10 μM ADP from 593 ± 56 nM to 360 ± 18 nM (SE, n=8).

These results indicate that conversion of arachidonic acid by the lipoxygenase pathway does not contribute to the rise in $[Ca^{2+}]_i$ evoked by this eicosanoid, and that the response is almost entirely accounted for by cyclooxygenase activity.

4. CONCLUSIONS

These results provide persuasive evidence that inhibition of 12-lipoxygenase does not suppress ADP-evoked Ca^{2+} signals in human platelets, and that 12-lipo-

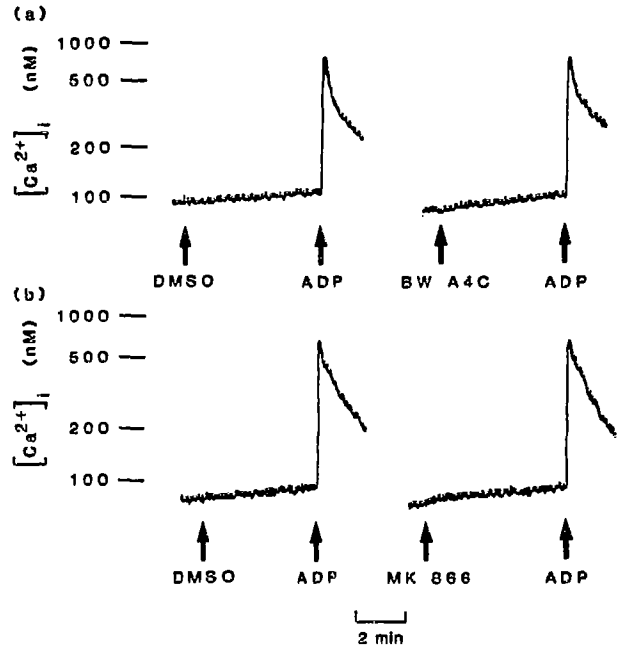

Fig. 3. Lack of effect of (a) 30 μM BW A4C and (b) 100 nM MK 866 on rises in $[Ca^{2+}]_i$ evoked by 10 μM ADP.

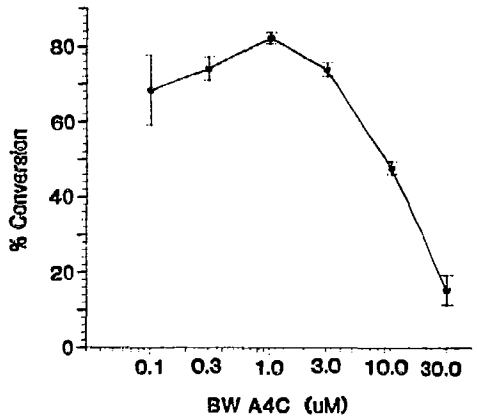

Fig. 4. Effect of BW A4C on conversion of [^{14}C]arachidonic acid to [^{14}C]12-HETE by platelet homogenate. Aliquots of the homogenate were incubated with the inhibitor for 5 min before the addition of [^{14}C]arachidonic acid. The reaction was allowed to proceed for 2 min at 37°C before termination with acid/acetone. Results are shown as percentage conversion of arachidonic acid to 12-HETE for duplicate samples.

xygenase products are not key steps in this signalling pathway. Furthermore, the present results emphasise the crucial role of cyclooxygenase products in Ca^{2+} signals evoked by arachidonic acid, and the virtual absence of Ca^{2+} responses to arachidonate when selective cycloxygenase inhibitors are present.

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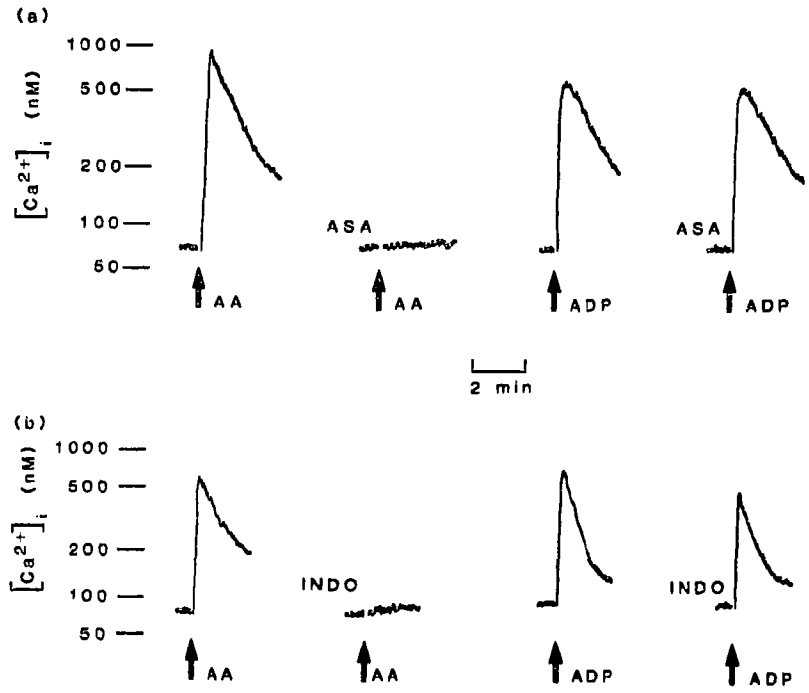

Fig. 5. Effects of cycloxygenase inhibition on responses to arachidonic acid and ADP. Effect of pretreatment for 15 min with (a) 100 μM aspirin (ASA) or (b) 15 μM indomethacin (INDO) on responses evoked by 10 μM arachidonic acid (AA) or 10 μM ADP.

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