

Thromboxane receptor stimulation inhibits adenylate cyclase and reduces cyclic AMP-mediated inhibition of ADP-evoked responses in fura-2-loaded human platelets

Stewart O. Sage and Johan W.M. Heemskerk

The Physiological Laboratory, University of Cambridge, Downingstreet, Cambridge, CB2 3EG, UK and Departments of Biochemistry and Human Biology, University of Limburg, 6200 MD Maastricht, The Netherlands

Received 23 December 1991

Stimulation of human platelets with the thromboxane A_2 analogue, U46619, after treatment with prostaglandin E_1 or forskolin, reduced the inhibition of ADP-evoked Mn^{2+} influx and the release of Ca^{2+} from intracellular stores. U46619 decreased the elevated concentration of 3',5'-cyclic AMP in platelets that were pretreated with prostaglandin E_1 . These results suggest that occupation of prostaglandin H_2 /thromboxane A_2 receptors, like those for other agonists, inhibits adenylate cyclase activity, which can contribute to the promotion of platelet activation.

Platelet; Thromboxane A_2 ; Adenylate cyclase; Calcium; Manganese; Fluorescent indicator

1. INTRODUCTION

Platelet activation by various agonists involves the production of stimulatory second messengers such as $Ins(1,4,5)P_3$, diacylglycerol and a rise in cytosolic calcium concentration ($[Ca^{2+}]_i$) [1,2]. Agonist-evoked activation is inhibited by elevation of the concentration of 3',5'-cyclic AMP (cAMP) following the stimulation of adenylate cyclase by compounds such as prostaglandins I_2 and E_1 (PGI_2 , PGE_1) and forskolin (see e.g. [3–5]).

It is well established that the platelet agonists thrombin, ADP, vasopressin and PAF-acether can inhibit adenylate cyclase (see e.g. [6–10]) as well as evoking the production of stimulatory messengers. Alpha-adrenergic agonists also inhibit the adenylate cyclase [11,12], apparently via the same inhibitory GTP binding protein, G_i , as other agents. In contrast, there is conflicting data concerning the effect of stimulation of prostaglandin endoperoxide PGH_2 /thromboxane A_2 (TxA_2) receptors on platelet adenylate cyclase. Several earlier and recent reports describe a lack of effect of the stable TxA_2 analogue, U46619, on basal or stimulated cyclase activity [13–16]. However, other earlier reports present evidence for an inhibition of PGE_1 -stimulated adenylate cyclase activity by TxA_2 [17] and U46619 [18]. Further conflict comes from the recent suggestion that

de-sensitisation of PGH_2/TxA_2 receptors leads to a sensitisation of platelet adenylate cyclase [19].

Here we have assessed the effects of the PGH_2/TxA_2 analogue, U46619, on the inhibition by PGE_1 or forskolin of ADP-evoked release of Ca^{2+} from intracellular stores and the entry of extracellular Ca^{2+} . We present evidence that U46619 reduces cAMP levels in PGE_1 -treated platelets and, thus, sensitises the cells for ADP-evoked calcium responses.

2. EXPERIMENTAL

Aspirinated, fura-2-loaded human platelets were prepared as previously described [20]. The cells were resuspended in a medium of composition (in mM): NaCl 145, KCl 5, $MgSO_4$ 1, HEPES 10, D-glucose 10, pH 7.4, at 37°C. Apyrase (20 $\mu g/ml$) was added. EGTA (1 mM) or 1 mM $CaCl_2$ and 200 μM $MnCl_2$ were added as required. Fura-2 fluorescence from a stirred platelet suspension, thermostated at 37°C, was recorded in a Cairn Spectrofluorimeter (Cairn Research, Sittingbourne, UK). For determination of $[Ca^{2+}]_i$, the ratio of fluorescence at excitation wavelengths of 340 and 380 nm were calibrated as described by Grynkiewicz et al. [21]. R_{max} was determined by addition of 50 μM digitonin in the presence of 1 mM external Ca^{2+} and R_{min} by the subsequent addition of 20 mM EGTA and 20 mM TRIS base. All signals were corrected for autofluorescence determined by adding 50 μM digitonin to cells in the presence of 10 mM Mn^{2+} .

The entry of divalent cations was determined from Mn^{2+} quenching of fura-2 fluorescence when 200 μM $MnCl_2$ was added to the external medium, and fluorescence monitored at the isobestic wavelength, 360 nm [22].

For determination of cAMP, 200 μl samples of platelet suspension ($3 \times 10^6/ml$) were added to 500 μl of ice-cold ethanol. Cyclic AMP was then extracted as described by Murray et al. [19] and assayed using a cAMP [^{125}I] radioimmunoassay kit (Rianen, Du Pont, Stevenage, UK).

PGE_1 (6 μM) or forskolin (2 μM) were added by 1,000-fold dilution from stocks in ethanol or DMSO, respectively. The vehicles were

Abbreviations: $[Ca^{2+}]_i$, cytosolic calcium concentrations; cAMP, 3',5'-cyclic AMP; PG, prostaglandin; TxA_2 , thromboxane A_2 .

Correspondence address: J.W.M. Heemskerk, Department of Biochemistry, University of Limburg, PO Box 616, 6200 MD Maastricht, The Netherlands.

without effect. Cells were incubated with inhibitors for 2 min before the first agonist addition.

ADP, apyrase, aspirin, PGE₁ and thrombin were from Sigma (Poole, UK). U46619 was from Upjohn (Kalamazoo, MI, USA). Forskolin and HEPES were from Calbiochem (La Jolla, CA, USA).

3. RESULTS AND DISCUSSION

3.1. Effects of U46619 and ADP on inhibition of internal Ca²⁺ release by cAMP

Fig. 1 shows the effect of PGE₁ (6 μ M) on the ability of ADP (40 μ M) and U46619 (0.5 μ M) to release Ca²⁺ from intracellular stores in fura-2-loaded human platelets. Fig. 1a shows rises in [Ca²⁺]_i evoked by the successive addition of ADP and U46619. Responses with the additions reversed are shown in Fig. 1b. After 2 min incubation with PGE₁, the response to ADP was almost abolished, but the subsequent addition of U46619 evoked a substantial rise in [Ca²⁺]_i (Fig. 1c). As shown in Fig. 1d, after pre-incubation with PGE₁, U46619 evoked almost no rise in [Ca²⁺]_i, but the response to a subsequent addition of ADP was partially restored. Similar results (not shown) were obtained when adenylyl cyclase was stimulated using its specific activator, forskolin (2 μ M) [23].

The ability of ADP to reduce the inhibition of the response evoked by U46619 by PGE₁ was as expected, since ADP is known to inhibit platelet adenylyl cyclase [8,9]. Since U46619 alleviated in part the inhibition of the ADP response, this agonist may have a similar inhibitory action on adenylyl cyclase.

3.2. Effects of U46619 and ADP on inhibition of Mn²⁺ entry by cAMP

To investigate agonist-evoked Ca²⁺ entry, fura-2-loaded platelets were stimulated in the presence of 1 mM external Ca²⁺ and 200 μ M Mn²⁺. Mn²⁺, known to enter the cells by the same pathways as Ca²⁺ [22,24,25], quenches the fluorescence of fura-2. Mn²⁺ entry can be monitored independently of changes in [Ca²⁺]_i by exciting the dye at the isobestic wavelength of 360 nm [21,22].

Fig. 2a shows the quenching of fura-2 fluorescence evoked by the addition of 40 μ M ADP, 0.5 μ M U46619 and 1 U/ml thrombin. (Thrombin was added to verify that there was quenchable dye remaining after the addition of the other agonists.) Fig. 2b shows the result with addition of U46619 before ADP. Pre-incubation of the platelets with 6 μ M PGE₁ for 2 min partially inhibited the Mn²⁺ entry evoked by ADP (40 μ M), whilst the entry evoked by subsequently added U46619 was hardly altered (Fig. 2c). In contrast, when U46619 was added first, the response was essentially abolished after PGE₁ pre-incubation, whilst the subsequent addition of ADP evoked a large fluorescence quench. Similar results were obtained using 2 μ M forskolin in place of PGE₁ (not shown).

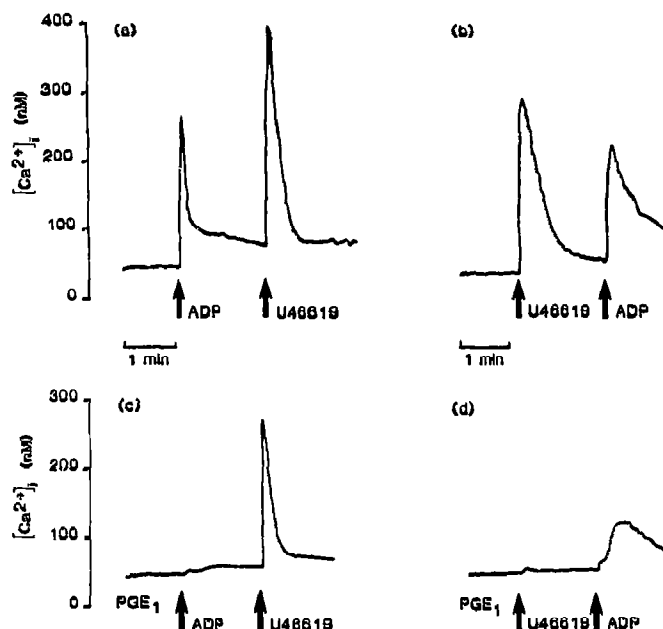


Fig. 1. Effects of ADP and U46619 on the inhibition of release of Ca²⁺ from intracellular stores by PGE₁. ADP (40 μ M) followed by U46619 (0.5 μ M), or vice versa, were added as shown to stirred suspensions of fura-2-loaded platelets in the presence of 1 mM EGTA. (a and b) Changes in [Ca²⁺]_i in untreated cells. (c and d) 6 μ M PGE₁ was added 2 min before the addition of the first agonist. Traces are typical of those obtained in 5–6 experiments.

The findings that PGE₁ and forskolin were less effective at inhibiting Mn²⁺ (Ca²⁺) entry evoked by ADP than by U46619 are in agreement with earlier studies. Stopped-flow fluorimetry indicates that the platelet ADP response consists of two phases of Ca²⁺ entry [25]. The first phase, apparently mediated by receptor-operated cation channels [26], is more resistant to inhibition by forskolin, whilst the delayed component, which may be related to the release of Ca²⁺ from intracellular stores, can be completely abolished using forskolin [20,25].

The Mn²⁺ quench experiments (Fig. 2) demonstrate the ability of ADP to reduce the inhibition of the U46619-evoked entry by PGE₁ and forskolin. It is also evident that prior stimulation by U46619 reduces the inhibition of ADP-evoked entry.

3.3. Effects of U46619 and ADP on cAMP in PGE₁-stimulated platelets

The simplest explanation of these results is that U46619, like ADP, is able to decrease the level of cAMP. We therefore determined platelet cAMP levels in experiments parallel to those with fura-2-loaded cells (see Fig. 1). The results are shown in Table 1. Prostaglandin E₁ evoked a 4–5-fold rise in cAMP. Two minutes after the addition of 40 μ M ADP, the level of cAMP was reduced to about 40% of its previous value and the subsequent addition of U46619 had no further effect. When U46619 was added as the first agonist after

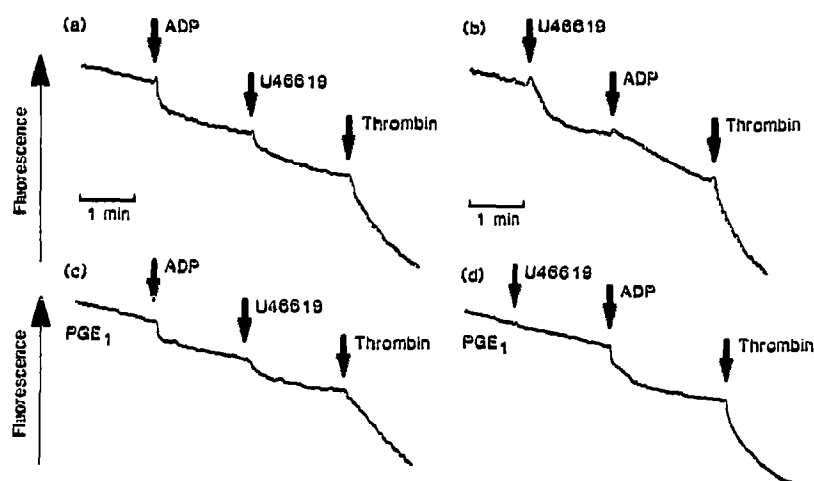


Fig. 2. Effects of ADP and U46619 on the inhibition of Mn^{2+} quenching of fura-2 fluorescence by PGE_1 . ADP ($40 \mu M$) followed by U46619 ($0.5 \mu M$), or vice versa, were added as shown to stirred suspensions of fura-2-loaded platelets in the presence of 1 mM external Ca^{2+} and $200 \mu M$ Mn^{2+} . Thrombin (1 U/ml) was added to indicate the availability of further quenchable fura-2. Changes in fura-2-fluorescence at the isobestic wavelength of 360 nm are shown. In (c) and (d), $6 \mu M$ PGE_1 was added 2 min before the addition of the first agonist. Traces are typical of those obtained in 5–6 experiments.

PGE_1 , the cAMP concentration was reduced to about 60% of its pre-addition value, and reduced still further by the subsequent addition of ADP. When the vehicles alone were added after PGE_1 , the concentration of cAMP continued to slowly rise (not shown).

The present data indicate that U46619, like other platelet agonists, can reduce cAMP levels in intact cells when elevated by PGE_1 or forskolin. Our results are in agreement with earlier reports that TxA_2 [17] and U46619 [18] inhibit PGE_1 -evoked rises in cAMP in platelet-rich plasma, although other reports [15,16] failed to demonstrate such an effect of thromboxane. Our results are also in agreement with the finding that the ability of PAF-acether to inhibit PGI_2 -stimulated cAMP levels was abolished by blockade of cyclooxygenase, suggesting that it is mediated by production

of TxA_2 [28]. Similarly, the ability of thrombin to inhibit adenylate cyclase has been suggested to be partly dependent on TxA_2 synthesis [16].

Recent reports show that U46619, in contrast to thrombin, is without effect [13] or gives only moderate reduction [14] of rises in cAMP evoked by PGE_1 . Although this at first glance seems to contrast with our results, it should be noted that we added U46619 after, rather than before, the agent used to stimulate adenylate cyclase. We also found that U46619 ($0.5 \mu M$) was less effective than ADP ($40 \mu M$) in lowering platelet cAMP levels (Table I) and in suppressing PGE_1 -induced inhibition of Ca^{2+} responses (Figs. 1 and 2). Together, this suggests that the coupling of the PGH_2/TxA_2 receptors to the cAMP metabolising complex is less effective than with thrombin [6,13,14] or ADP [9].

It has been reported that de-sensitization of the PGH_2/TxA_2 receptor with U46619 enhances adenylate cyclase stimulation by the PGI_2 analogue, iloprost, and by forskolin [19]. This effect was seen after 30 min de-sensitization, which may account for the opposite effect of U46619 observed in our experiments. However, de-sensitisation to U46619 is reported to be rapid, with a half-time of 2–3 min [27]. Since we find decreased and not increased cAMP levels after 2 min treatment with U46619, sensitisation of the cyclase appears not to be the dominant effect under the conditions of our experiments.

At present we do not know how U46619 reduces platelet cAMP levels, although it is possible that it inhibits adenylate cyclase activity via G_i , like other agonists [6–10]. An alternative possibility might be the activation of a cyclic nucleotide phosphodiesterase, accelerating the breakdown of cAMP. Additionally, it has

Table I

Effects of ADP and U46619 on PGE_1 -induced cyclic AMP levels

Addition	cAMP (pmol/ 10^8 platelets)	Addition	cAMP (pmol/ 10^8 platelets)
Expt. A		Expt. B	
EGTA	8 ± 1	EGTA	7 ± 1
PGE_1	34 ± 4	PGE_1	37 ± 6
ADP	13 ± 1	U46619	22 ± 4
U46619	15 ± 3	ADP	11 ± 1

Platelets stirred at $37^\circ C$ were supplied, successively, with EGTA (1 mM), PGE_1 ($6 \mu M$), ADP ($40 \mu M$) and U46619 ($0.5 \mu M$) (Experiment A) or with EGTA, PGE_1 , U46619 and ADP (Experiment B) at 2 min intervals. Aliquots were taken just before each addition, and cAMP determined as described in section 2. Data are mean values \pm S.E. ($n=3$). Calcium responses in fura-2-loaded platelets from the same populations, checked in parallel experiments, were similar to those in

Fig. 1.

been reported that activation of protein kinase C by phorbol esters results in the inhibition of platelet adenylate cyclase [28,29]. However, this effect is not large and occurs with greater protein kinase C activation than is achieved with the physiological agonists thrombin and PAF [30]. The contribution of protein kinase C to the inhibitory effects of U46619 reported here is, therefore, likely to be small.

Whatever the mechanism, the ability of $\text{PGH}_2/\text{TxA}_2$ receptor occupation to decrease platelet cAMP levels, along with the similar actions of other agonists, would be of physiological significance in promoting activation, as we have shown for the ADP-evoked Ca^{2+} signal.

Acknowledgements: This work was supported in part by The Netherlands Organisation for Scientific Research (NWO). SOS held a Royal Society 1983 University Research Fellowship. We thank W. David Clarkson for participation in some experiments.

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