Concomitant activation of G_i and G_q protein-coupled receptors does not require an increase in cytosolic calcium for platelet aggregation

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Abstract U46619 is a potent platelet agonist, its binding to the thromboxane A_2 receptor resulting in G_q-binding protein-mediated responses; nevertheless, it is unable to cause platelet aggregation, unless released ADP is present. In this study we demonstrate that G_i activation is the step U46619 lacks to cause platelet aggregation; in fact, when platelets were treated with an ADP scavenger system, the response to U46619 was restored by the addition of epinephrine, which activates platelets via a G_i protein. The concomitant activation of G_i and G_q proteins does not require increased cytosolic calcium to cause aggregation, as assessed by the fact that platelets treated with the intracellular calcium chelator BAPTA were able to respond to U46619 provided ADP or epinephrine was present. Moreover, as the calcium ionophore ionomycin, at low concentrations, potentiated the response to U46619 but not to epinephrine, we may conclude that calcium influx preferentially activates a G_i downstream signalling pathway.

Key words: Platelet aggregation; U46619; G protein; Calcium influx

1. Introduction

Platelet aggregation is crucial for primary hemostasis and for the formation of platelet rich thrombi which could occlude blood vessels in arterial diseases. During the last decade great interest has been shown in focusing cell surface receptors involved in platelet activation as well as in defining the role of heterotrimeric G proteins in mediating the interaction of such receptors with downstream intracellular effectors [1]. Recently, the existence of two distinct G protein-coupled ADP receptors on platelets, one (P2Y1) coupled to phospholipase C, involving G_q, and the other ($P2T_{AC}$) to inhibition of adenylyl cyclase, via a G_i-mediated event, has been demonstrated [2], their concomitant activation being essential for ADP-induced platelet aggregation [3].

These data are in agreement with those of Savi et al. who demonstrated that the inhibition of platelet aggregation by A3P5PS, an antagonist of the G_q-coupled ADP receptor (P2Y1), was restored by the addition of serotonin, a non-aggregating compound capable of stimulating G_q-coupled phospholipase C activation [4].

In a previous work we demonstrated that the thromboxane A_2 synthetic agonist, U46619, which causes the selective activation of G_q without affecting the adenylyl cyclase pathway [5], was not able to elicit fibrinogen receptor exposure unless ADP was present [6]. The same results were obtained in platelets degranulated by means of thrombin and reexposed to agonists [7,8]. The aim of this study was thus to verify whether G_i activation is the step U46619 lacks to cause platelet aggregation. For this purpose we studied platelets treated with an ADP scavenger system subsequently stimulated with U46619 plus epinephrine, an agonist which is capable of selectively activating the G_i protein and of potentiating platelet aggregation induced by other agonists which activate G_q [9,10].

Moreover, since some authors have suggested that among the events related to the G_q-coupled ADP receptor, cytosolic calcium increase is a major event for aggregation [11], while we have demonstrated that the exposure of the platelet fibrinogen binding site could occur independently of an increase in cytosolic Ca^{2+} [8], this study was also aimed at delineating the role of calcium in platelet aggregation in cells treated with the intracellular calcium chelating agent BAPTA.

2. Materials and methods

Blood samples were collected in acid/citrate/dextrose (ACD) from informed healthy volunteers who denied having taken any drugs in the 2 weeks before blood sampling.

Platelet rich plasma (PRP) obtained after centrifugation ($180\times g$ for 15 min) was further centrifuged ($800\times g$ for 20 min) to concentrate the platelets (6×10^8 platelets/ml). The concentrated platelets were incubated for 15 min at 37°C with 1 mM acetylsalicylate (Sigma, St. Louis, MO, USA) and then gel filtered on Sepharose 2B-CL (Pharmacia, Uppsala, Sweden) using Ca^{2+}-free Tyrode's buffer containing 0.2% albumin (bovine serum fraction V-BSA), 0.1% glucose and 10 mM HEPES (pH 7.35) (all from Sigma).

In some experiments gel filtered platelets (GFP) were treated with the ADP scavenger system creatine phosphate/creatine kinase (CP/CPK) (Sigma) before the addition of agonists.

2.1. Preparation of thrombin degranulated platelets (TDP)

The preparation of TDP has been previously described [7]. Briefly, before thrombin activation (0.5 U/ml of human α-thrombin, generous gift of Dr. J.W. Fenton II, NY State Department of Health, Albany, NY, USA), the GFP were incubated for 5 min at 37°C with 0.1 mM Arg-Gly-Asp-Ser (RGDS), 0.12 mM Gly-Pro-Arg-Pro (GPRP), 20 mM creatine phosphate (CP), 50 U/ml creatine kinase (CPK) and 0.2 mM $CaCl_2$. After 1 min tosyl-arginyl-methyl ester (TAMe) (10 mM) and hirudin (10 mU/ml) were added as thrombin inhibitors and ACD (40 μl/ml of GFP) was used to lower the pH of the suspension to 6.5 (all the products were from Sigma). The platelets were then washed twice by centrifugation at $800\times g$ for 10 min and finally resuspended in Tyrode's buffer.

2.2. Preparation of BAPTA-treated platelets

The concentrated platelets were incubated for 30 min at 37°C with 1 mM aspirin, 100 μM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid acetoxymethyl ester (BAPTA-AM) and 3 μM Fura-2-AM (both from Molecular Probes, Eugene, OR, USA). Excess BAPTA-AM and Fura-2-AM were separated from the platelets by

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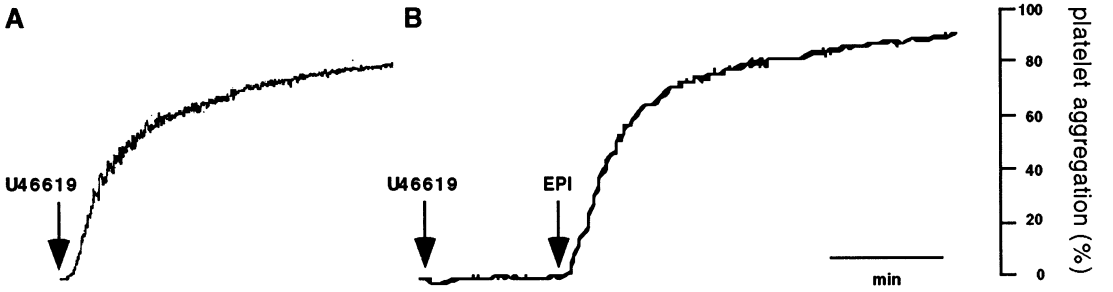

Fig. 1. Platelet aggregation in response to U46619 (2 μM) plus epinephrine (5 μM) in acetylsalicylate-treated (1 mM) GFP in the presence of the ADP scavenger system CP/CPK (40 mM/100 U/ml) (B). A: Control, untreated GFP in response to U46619 (2 μM). The figure is representative of five experiments performed.

gel filtration. On each preparation changes in intracellular calcium concentrations in response to thrombin (1 U/ml), U46619 (2 μM) and ADP (20 μM) were evaluated. The fluorescence changes were monitored with a Kontron SFM 25 fluorimeter, set at 340 nm excitation and 510 nm emission. Intracellular free calcium was calibrated according to Grynkiewicz [12]. If the ΔnM of Ca^{2+} obtained in response to each agonist was higher than 10 the platelet suspension was not employed.

2.3. Platelet aggregation

In vitro platelet aggregation was evaluated according to Born [13] in a four sample PACKS-4 (Helena Laboratories, Beaumont, TX, USA) aggregometer using siliconized glass cuvettes at 37°C under continuous stirring. Fibrinogen (1 mg/ml) (Sigma) was added before the agonists.

3. Results and discussion

It has recently been demonstrated that ADP-induced platelet aggregation is dependent on the coactivation of two different G protein-coupled receptors, one (P2Y1) coupled to the activation of G_q and the other ($P2T_{AC}$) coupled to G_i [3]. The requirement of converging signalling pathways from the two different proteins might explain the inability of the thromboxane A_2 agonist U46619 to evoke per se an aggregometric response in the absence of released ADP [6,8]. To test the validity of this hypothesis we added epinephrine, which promotes inhibition of adenylyl cyclase by coupling to G_i, to platelets stimulated with U46619. The results show that the delayed addition of epinephrine (5 μM), which was not able to induce platelet aggregation (data not shown), to a suspension of GFP treated with the ADP scavenger system CP/CPK (40 mM/100 U/ml) exposed to U46619 (2 μM) resulted in maximal aggregation (74.0 ± 6.6%), comparable to that obtained in control GFP activated with U46619 (Fig. 1).

Savi et al. [4] have proposed that P2Y1-mediated phospholipase C activation through a G_q-coupled event and the subsequent cytosolic calcium increase might be a major event in platelet aggregation. In order to investigate the role exerted by calcium ions in this platelet response we examined a platelet preparation treated with the intracellular calcium chelating agent BAPTA in the absence of calcium in the external medium. This platelet suspension was preincubated with minimal concentrations of CP/CPK (0.4 mM/1 U/ml) since U46619-induced ADP release was lower than 10% (unpublished

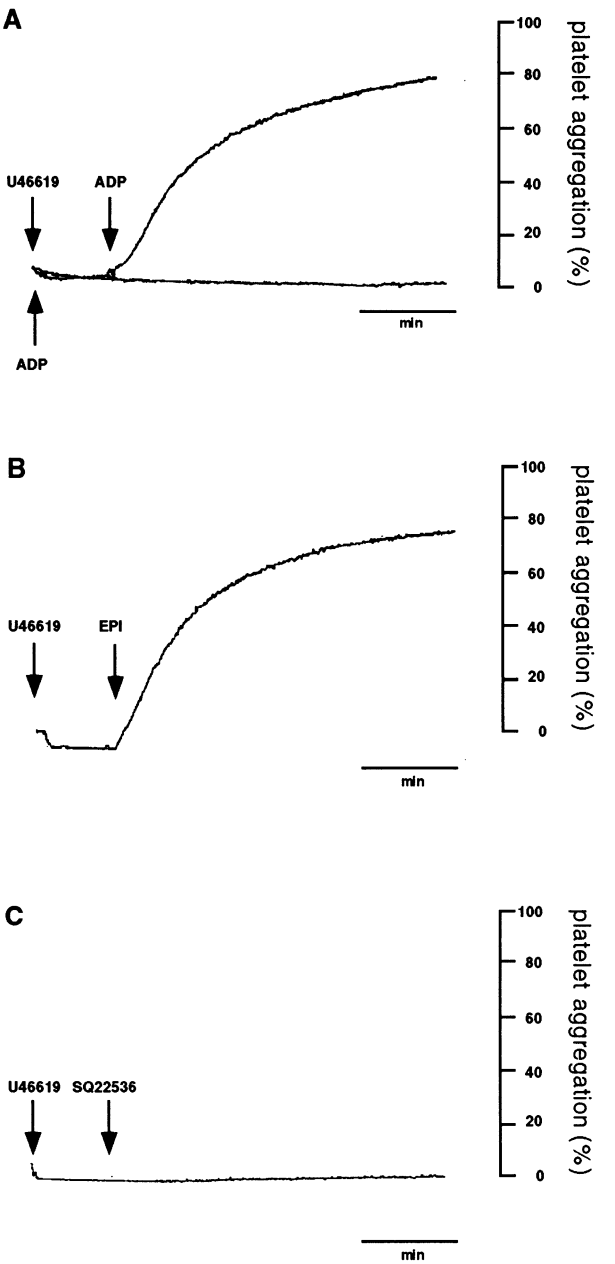

Fig. 2. Platelet aggregation patterns in response to ADP (5 μM) (A, lower curve), U46619 (2 μM) plus ADP (A, upper curve), U46619 (2 μM) plus epinephrine (5 μM) (B) and U46619 (2 μM) plus SQ22536 (10 μM) (C) in acetylsalicylate-, BAPTA-treated (1 mM and 100 μM respectively) platelets in the presence of low concentrations of CP/CPK (0.4 mM/1 U/ml). The figure is representative of five experiments.

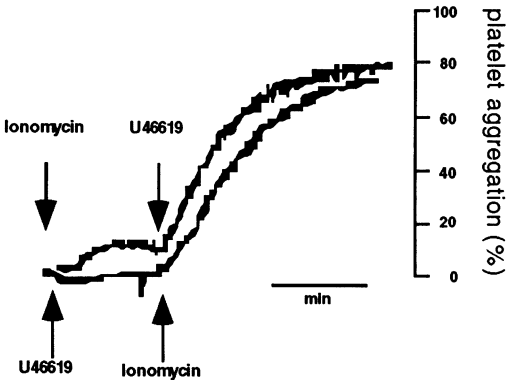

Fig. 3. Platelet aggregation patterns in response to U46619 (2 μM) plus ionomycin (0.5 μM) in acetylsalicylate-treated (1 mM) GFP, in the presence of CP/CPK (40 mM/100 U/ml). The calcium ionophore was added either before (upper curve) or after (lower curve) U46619. The figure is representative of four experiments performed.

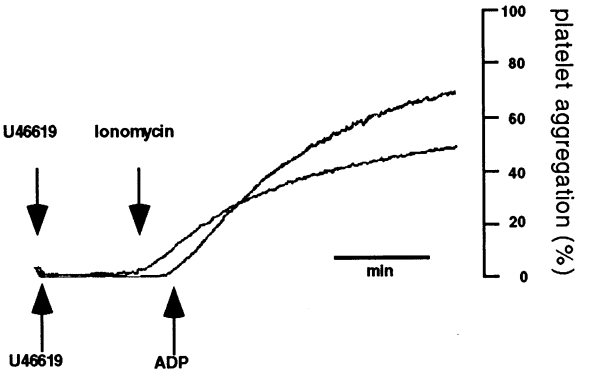

Fig. 4. Platelet aggregation patterns in response to U46619 (2 μM) plus ADP (5 μM) or ionomycin (0.5 μM) in TDP treated with low concentrations of CP/CPK (0.4 mM/1 U/ml). The figure is representative of four experiments performed.

data). Fig. 2 shows that a full aggregation was achievable in platelets activated with U46619 (2 μM) plus ADP (5 μM) or epinephrine (5 μM) (87.4 ± 11% and 76.8 ± 14% respectively) (panels A and B), while the single agonists did not cause any response (panel A). Since both ADP and epinephrine exert their action by inhibiting the adenylyl cyclase responsible for the production of adenosine 3′,5′-cyclic monophosphate (cAMP), we hypothesized that the reduction of cAMP levels might be the necessary condition for U46619-induced platelet aggregation to occur. For this purpose the aggregometric response to SQ22536 (10 μM), a drug which inhibits cytosolic adenylyl cyclase [14], used in combination with U46619 was evaluated. The results showed that BAPTA-treated GFP did not respond to this combined stimulation clearly indicating that the reduction of cAMP concentrations is not the mechanism required by U46619 to induce aggregation (Fig. 2C).

Offermanns et al. [15] demonstrated that high concentrations (100 μM) of the calcium ionophore A23187 caused a dramatic secretion followed by aggregation in $G\alpha_q$-deficient mouse platelets, indicating that calcium influx is able to activate a downstream signalling molecule in the two pathways involving both G_q and G_i. However, this activation seems to be triggered only by a massive calcium influx through the plasma membrane, since, when we tested the effects of low concentrations of another calcium ionophore, ionomycin (0.5 μM), on a suspension of control platelets treated with CP/CPK (40 mM/100 U/ml), we only observed a reversible wave of aggregation (16.5 ± 5.3%) (Fig. 3). Nevertheless, when ionomycin was used synergistically with U46619, it mimicked the response to epinephrine in potentiating U46619-induced activation, giving rise to full aggregation (88.9 ± 7.6%) (Fig. 3). This response was unmodified if ionomycin was added before U46619.

A similar mechanism of amplification was observed in TDP in which the ADP stored in the granules had been released; in fact, platelet aggregation to U46619 was restored by the addition of ionomycin (0.5 μM) similarly to what we observed after the addition of ADP (5 μM) (48.6 ± 3.5% and 67.3 ± 4.5% respectively) (Fig. 4).

Conversely, when tested in BAPTA-treated platelets, ionomycin, which caused a slight response (23.4 ± 3.7%) when used at high concentrations (5 μM) in the presence of $CaCl_2$ 1mM (Fig. 5A), was unable to amplify platelet response to U46619 (2 μM) unless calcium was present, the maximum effect being achieved in the presence of 1 mM $CaCl_2$ (Fig. 5B). The fact that ionomycin is unable to amplify platelet response to epinephrine, which promotes the inhibition of adenylyl cyclase by a G_i-coupled mechanism, both in platelets treated with the ADP scavenger system and in BAPTA-treated platelets (data not shown), might be explained by assuming that entry of a small concentration of calcium activates a downstream biochemical pathway coupled to G_i.

Therefore, from our results we can conclude that the coactivation mechanism of two different subtypes of GTP binding

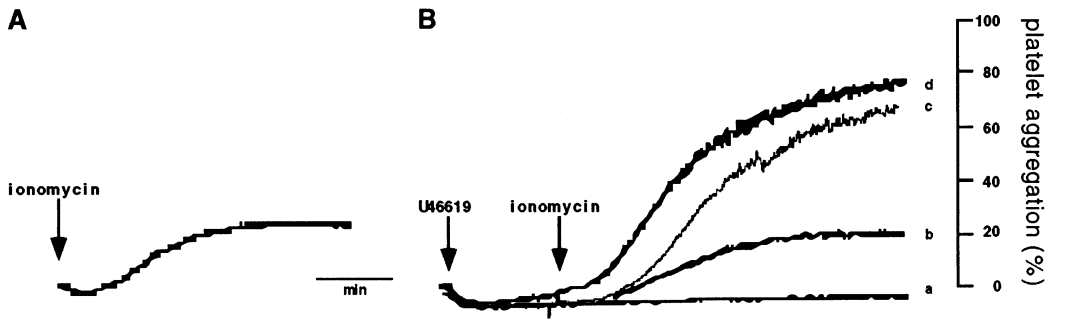

Fig. 5. Platelet aggregation patterns in response to ionomycin (5 μM) in the presence of $CaCl_2$ 1 mM (A) and to ionomycin (2 μM) plus U46619 (2 μM) in the absence (a) and in the presence of increasing concentrations of $CaCl_2$ (b: 0.2 mM; c: 0.5 mM; d: 1 mM) in acetylsalicylate-, BAPTA-treated (1 mM and 100 μM respectively) platelets. The figure is representative of six experiments performed.

proteins, G_i and G_q, may be required for U46619-induced platelet aggregation, and that the activation of these two G proteins does not require any increase in cytosolic calcium concentration.

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