Concomitant activation of G_i protein-coupled receptor and protein kinase C or phospholipase C is required for platelet aggregation

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Abstract **It has recently been suggested that the concomitant activation of two distinct G protein-coupled receptors (G_i and G_q) is essential for platelet aggregation: in fact, the thromboxane A_2 synthetic agonist, U46619, which causes the selective activation of G_q, is not able to elicit fibrinogen receptor exposure unless ADP or epinephrine is present. In the present study we demonstrate that a direct G_q activation is not required for platelet aggregation and that the activation of an enzyme downstream of G_q, such as phospholipase C (PLC) or protein-kinase C (PKC), is sufficient for such a process. In fact, platelet aggregation occurred in response to the snake venom toxin convulxin, which activates the PLC isoform PLCγ2 or to cytosolic PKC activator phorbol 12-myristate 13-acetate (PMA) provided a G_i protein-coupled receptor was activated by ADP or epinephrine. The evidence that the PKC inhibitor, Ro 31-8220 did not suppress platelet aggregation in response to convulxin plus ADP or epinephrine led us to conclude that PLC and PKC are both involved in platelet aggregation, although not concomitantly, provided a G_i protein-coupled receptor is activated.**

Key words: Platelet aggregation; PKC; PLC; G-protein

1. Introduction

The integrin αIIbβ3-ligand interactions have been widely studied due to their crucial role in the adhesive events critical for primary hemostasis and formation of the hemostatic plug. The fibrinogen binding site on αIIbβ3, which in resting platelets is inaccessible to fibrinogen, undergoes a conformational change once platelets are stimulated, thus permitting the formation of a stable aggregate. Many authors have tried to identify the cell surface receptors involved in platelet aggregation and to define the downstream intracellular effectors responsible for the exposure of the fibrinogen binding site [1]. Recently, it has been suggested that the concomitant activation of two distinct G protein-coupled ADP receptors, one (P2Y1) coupled to phospholipase C, involving G_q, and the other ($P2T_{AC}$) to inhibition of adenylyl cyclase, via a G_i-mediated pathway [2] is essential for ADP-induced platelet aggregation [3,4]. This is in agreement with the results of a previous work in which we demonstrated that the thromboxane A_2 synthetic agonist, U46619, which causes the selective activation of G_q [5], was not able to elicit fibrinogen binding unless ADP was released [6].

Subsequently, in order to confirm this theory, we performed experiments on platelets treated with an ADP scavenger system and stimulated with U46619 plus epinephrine, an agonist which selectively activates the G_i protein and potentiates platelet aggregation induced by other agonists acting via G_q [7,8]. The results demonstrated that the coactivation of G_i and G_q was essential for U46619-induced platelet aggregation, and did not require any increase in cytosolic calcium concentration [9].

Therefore, aim of the present study was to verify whether a direct G_q activation is required for platelet aggregation or whether a downstream enzyme activated by G_q, such as phospholipase C (PLC) or protein-kinase C (PKC), is sufficient for such a process.

For this purpose we used phorbol 12-myristate 13-acetate (PMA) which is a cytosolic PKC activator, and convulxin, a non-enzymic snake venom toxin from *Crotalus durissus terrificus*, which is a selective agonist of the collagen receptor GpVI. This toxin activates the PLC isoform PLCγ2 and induces tyrosine phosphorylation not requiring protein G_q activation and independently of integrin αIIbβ3 [10,11].

Moreover, this study was aimed at better defining the role of ADP release reaction in platelet aggregation induced by PMA and convulxin.

The results support the idea that the activation of PLC or PKC is sufficient to cause platelet aggregation only in the presence of a concomitant activation of a G_i protein-coupled receptor.

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 prior to blood sampling. Platelet Rich Plasma (PRP) was obtained after centrifugation ($180\times g$ for 15 min) and then 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 aspirin (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) (20 mM/ 50 U/ml) (Sigma) before the addition of the agonists.

Convulxin from *Crotalus durissus terrificus* (Latoxan, Rosans, France) was purified according to Polgar et al. [11].

2.1. Platelet aggregation

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

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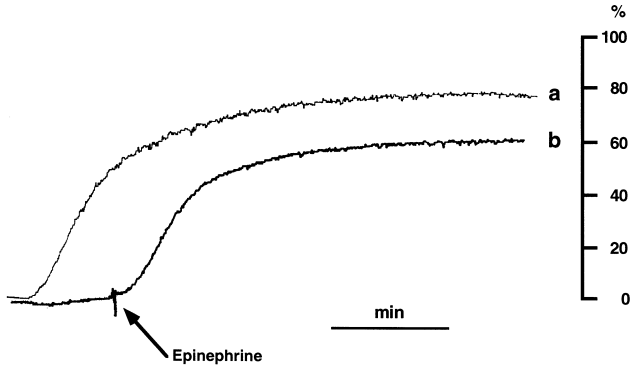

Fig. 1. Platelet aggregation in aspirin-treated (1 mM) gel-filtered platelets in response to convulxin (5 ng/ml) alone (a) or plus epinephrine (5 μM) in the presence of the ADP scavenger system CP/CPK (20 mM/50 U/ml) (b). The figure is representative of five experiments performed.

2.2. Changes in intracellular calcium concentrations

The changes in intracellular calcium concentrations were monitored in gel-filtered platelets using the fluorescent dye Fura-2-AM (3 μM) (Molecular Probes, Eugene, OR, USA) in response to convulxin (5 ng/ml). 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].

2.3. 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-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) (Molecular Probes). Excess BAPTA-AM was separated from the platelets by gel-filtration. On each preparation the changes in intracellular calcium concentrations were tested. In the presence of a ΔnM of Ca^{2+} obtained in response to convulxin higher than 10, the platelet suspension was not used.

3. Results

In order to investigate the capability of convulxin and PMA to induce platelet aggregation without the synergistic action of the amplification mechanisms, all the experiments were performed in the presence of aspirin. Moreover, in those experiments in which a complete inhibition of ADP release was required, the platelets were further treated with the ADP scavenger system creatine phosphate/creatine kinase (CP/CPK).

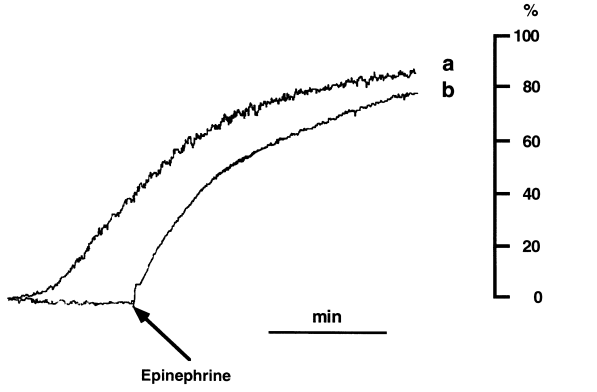

Fig. 2. Platelet aggregation patterns in aspirin-treated (1 mM) gel-filtered platelets in response to PMA (10 μM) alone (a) or in combination with epinephrine (5 μM) in the presence of the ADP scavenger system CP/CPK (20 mM/50 U/ml) (b). The figure is representative of five experiments performed.

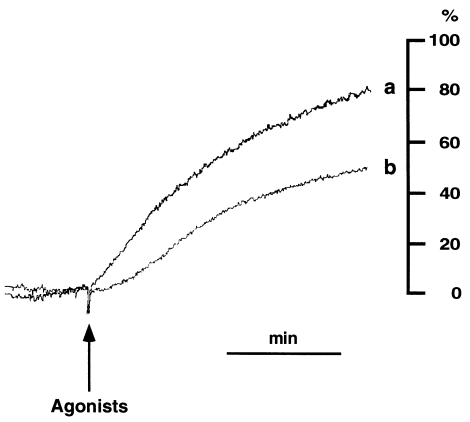

Fig. 3. Platelet aggregation patterns in response to convulxin (5 ng/ml) plus ADP (5 μM) (a) or epinephrine (5 μM) (b) in aspirin-, Ro 31-8220-treated (1 mM and 10 μM respectively) gel-filtered platelets. The figure is representative of five experiments performed.

Fig. 1 shows platelet aggregation induced by convulxin (5 ng/ml) in control (a) and in CP/CPK (20 mM and 50 U/ml, respectively) treated platelets, subsequently stimulated with epinephrine (5 μM) (b). The results show that convulxin is not able to induce platelet aggregation, monitored for at least 5 min (data not shown), in platelets in which released ADP was removed by the scavenger system CP/CPK, unless low concentrations of epinephrine (5 μM), unable per se to cause any aggregometric response, were added.

Fig. 2 shows PMA-induced (10 μM) platelet aggregation in control (a) and CP/CPK treated platelets, to which epinephrine (5 μM) (b) was added. The results show that platelet aggregation in response to PMA is dependent on the ADP released by the granules, as demonstrated by the fact that CP/CPK treatment prevented the aggregation that was restored only by the addition of the second agonist.

In order to discriminate the different roles played by PKC and PLC in this process we employed the selective inhibitor of PKC Ro 31-8220. In this experimental model the use of CP/CPK was not required, as a total inhibition of the degranulation was achieved with Ro 31-8220 alone [6].

Fig. 3 shows the aggregometric patterns of platelets treated with Ro 31-8220 (10 μM), activated with convulxin (5 ng/ml), plus epinephrine (5 μM) or ADP (5 μM). The results demonstrate that both epinephrine and ADP are able to induce

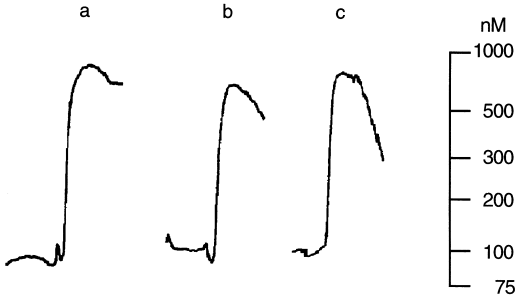

Fig. 4. Changes in intracellular calcium concentrations in control (a), CP/CPK treated (20 mM/50 U/ml) (b) and Ro 31-8220 treated (10 μM) (c) Fura-2 loaded gel-filtered platelets in response to convulxin (5 ng/ml). The figure is representative of five experiments performed.

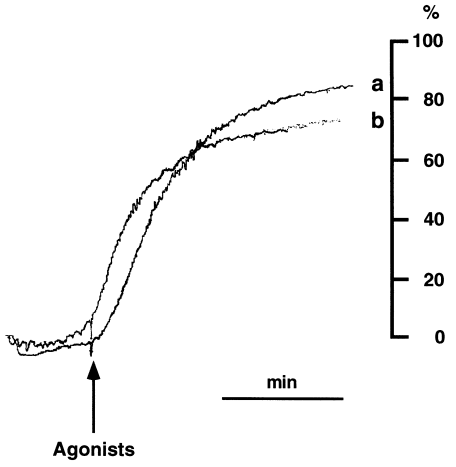

Fig. 5. Platelet aggregation patterns in response to convulxin (5 ng/ml) in combination with ADP (5 μM) (a) or epinephrine (5 μM) (b) in aspirin-, BAPTA-treated (1 mM and 100 μM respectively) platelets. The figure is representative of five experiments performed.

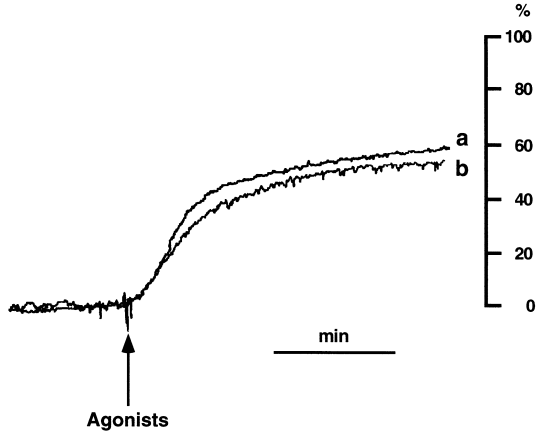

Fig. 7. Platelet aggregation patterns in response to PMA (10 μM) plus ADP (5 μM) (a) or epinephrine (5 μM) (b) in aspirin-, BAPTA-treated (1 mM and 100 μM respectively) platelets. The figure is representative of five experiments performed.

platelet aggregation synergistically with convulxin through biochemical events independent of protein-kinase C activation. In the presence of Ro 31-8220 no aggregometric response to ADP stimulation was observed (data not shown).

As expected, in the presence of PKC inhibition, PMA proved unable to induce platelet aggregation in spite of epinephrine or ADP addition (data not shown).

Fig. 4 reports the changes in intracellular calcium concentrations in convulxin-stimulated platelets in the presence or absence of the ADP scavenger system CP/CPK or of the PKC inhibitor Ro 31-8220. The results show that convulxin was able to induce an appreciable increase in cytosolic calcium concentrations (trace a), that was not modified by the treatment with CP/CPK (b) or Ro 31-8220 (c).

As in a previous work we demonstrated that a calcium chelator, BAPTA, did not cause any inhibition of platelet aggregation in response to U46619 plus ADP or epinephrine [9], we tested the role of calcium in convulxin- and PMA-induced platelet aggregation.

Fig. 5 shows the aggregometric patterns of BAPTA-treated platelets in response to convulxin (5 ng/ml) followed by the addition of epinephrine (5 μM) or ADP (5 μM). The results demonstrate that convulxin was able to cause platelet aggregation only if the degranulation reaction, with the subsequent release of endogenous ADP, took place; in fact, the addition of ADP or epinephrine to convulxin-stimulated platelets gave rise to a maximal aggregation.

In this process PKC activation does not seem an essential prerequisite, even in the absence of calcium. In fact, when BAPTA-treated platelets were incubated with the PKC inhibitor Ro 31-8220 (10 μM) (Fig. 6) before the stimulation with convulxin, a relevant response was obtained after the addition of epinephrine or ADP.

Comparable results were obtained when PMA was used as an agonist (Fig. 7).

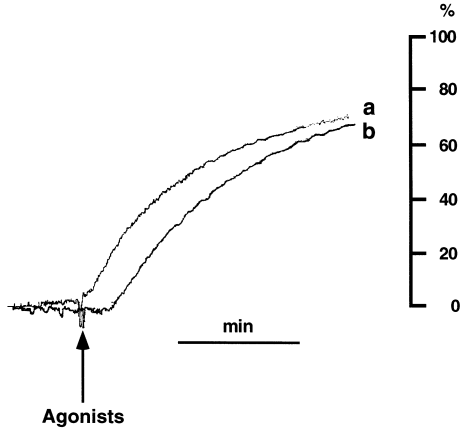

Fig. 6. Platelet aggregation patterns in response to convulxin (5 ng/ml) in combination with ADP (5 μM) (a) or epinephrine (5 μM) (b) in aspirin-, BAPTA-treated (1 mM and 100 μM, respectively) platelets incubated with Ro 31-8220 (10 μM). The figure is representative of five experiments performed.

4. Discussion

Platelet aggregation requires the concomitant activation of two G-protein coupled receptors, G_i and G_q [3,4,9]. In a previous work we demonstrated that the synthetic agonist of thromboxane A2 receptor U46619, which activates G_q protein, was per se unable to cause platelet aggregation without the concomitant activation of a G_i protein coupled receptor induced by ADP or epinephrine [5,8]. G_i activation is independent of reduction of cyclic AMP concentration, as demonstrated by the fact that platelets pre-treated with the adenylate cyclase inhibitor SQ 22536 failed to aggregate in response to U46619 alone [9].

The results shown in this paper demonstrate that a pathway downstream of G_q activation is able to cause the conformational changes in the integrin αIIbβ3 responsible for platelet aggregation, provided a G_i protein coupled receptor is also activated. In fact, by selectively activating the isoform of phospholipase C PLCγ2, with the snake venom convulxin, which is independent of G_q protein, but dependent on tyrosine kinase activation [10,11], we observed a platelet activation similar to that obtained in response to U46619. Gel-filtered

platelets aggregated in response to convulxin only in the presence of ADP released from platelet granules. When ADP was removed by means of the scavenger system CP/CPK, an appreciable aggregometric response was achieved only when a G_i protein coupled receptor, like the α2-adrenergic receptor, was rapidly activated [7,8].

Platelet pre-treatment with CP/CPK did not inhibit platelet activation induced by convulxin, as demonstrated by the observation that such treatment did not modify the changes in intracellular calcium concentrations. These results led us to hypothesize that the activation of PLC or other biochemical pathways triggered by the G_q protein are responsible for platelet aggregation.

The initial hypothesis conferring to protein kinase C activation the role of key step in G_i protein dependent platelet aggregation was based on the observation that the cytosolic protein kinase C activator PMA behaved in the same manner as U46619 and convulxin, requiring epinephrine or ADP to induce platelet aggregation. On the contrary, platelet treatment with the protein kinase C inhibitor Ro 31-8220 failed to inhibit the synergistic response of convulxin plus ADP or epinephrine. This datum is in agreement with previous observations indicating that Ro 31-8220 did not modify the aggregometric response to U46619 plus ADP or to thrombin alone [6,13]. From our results we can thus conclude that both phospholipase C and protein kinase C are required for platelet aggregation, although their concomitant activation is not necessary. Moreover, the pathway involved in this process is not dependent on calcium concentration, as platelet treatment with the cytosolic calcium chelator BAPTA did not inhibit the aggregometric response to the combined stimulation by convulxin or PMA plus ADP or epinephrine. Besides, in this platelet preparation, PLC activation was sufficient to induce a complete aggregation in response to convulxin plus ADP or epinephrine and did not require the concomitant activation of PKC, as demonstrated by the fact that the protein kinase C inhibitor Ro 31-8220 failed to inhibit this response.

From all our data we can conclude that PLC and PKC are both involved in inducing platelet aggregation, their concomitant activation not being necessary, provided a G_i protein-coupled receptor is activated.

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References

[1] Shattil, S.J., Gao, J. and Kashiwagi, H. (1997) Thromb. Haemost. 78, 220–225.
[2] Daniel, J.L., Dangelmaier, C., Jin, J., Ashby, B., Smith, J.B. and Kunapuli, S.P. (1998) J. Biol. Chem. 273, 2024–2029.
[3] Jin, J. and Kunapuli, S.P. (1998) Proc. Natl. Acad. Sci. USA 95, 8070–8074.
[4] Savi, P., Beauverger, P., Labouret, C., Delfaud, M., Salel, V., Kaghad, M. and Herbert, J.M. (1998) FEBS Lett. 422, 291–295.
[5] Ohkubo, S., Nakata, N. and Ohizumi, Y. (1996) Br. J. Pharmacol. 117, 1095–1104.
[6] Pulcinelli, F.M., Ashby, B., Gazzaniga, P.P. and Daniel, J.L. (1995) FEBS Lett. 364, 87–90.
[7] Lanza, F., Beretz, A., Stierle, A., Hanau, D., Kubina, M. and Cazenave, J.P. (1988) Am. J. Physiol. 255, 1276–1288.
[8] Steen, V.M., Holmsen, H. and Aarbakke, G. (1993) Thromb. Haemost. 70, 506–513.
[9] Pulcinelli, F.M., Pesciotti, M., Pignatelli, P., Riondino, S. and Gazzaniga, P.P. (1998) FEBS Lett. 435, 115–118.
[10] Francischetti, I.M.B., Carlini, C.R. and Guimaraes, J.A. (1998) Arch. Biochem. Biophys. 354, 255–262.
[11] Polgar, J., Clemetson, J.M., Kehrel, B.E., Wiedemann, M., Magnenat, E.M., Wells, T.N.C. and Clemetson, K.J. (1997) J. Biol. Chem. 272, 13576–13583.
[12] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3445.
[13] Walker, T.R. and Watson, S.P. (1993) Biochem. J. 289, 277–282.