Prostaglandin E₁ and forskolin antagonize C-kinase activation in the human platelet

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In human platelets, prostaglandin E₁ and forskolin inhibit 5-hydroxytryptamine-induced phospholipase C, C-kinase and myosin light-chain kinase activity in a concentration-dependent way. Phospholipase C activation, however, was only partly inhibited, and this at higher concentrations than the protein kinases. Direct activation of the C kinase either by exogenous synthetic diacylglycerol or by 12-*O*-tetradecanoylphorbol 13-acetate was antagonized by prostaglandin E₁ and forskolin. Since C-kinase activation is one of the key events in excitatory signal transduction in the platelets, we suggest that the inhibitory effect of agents that increase platelet cyclic AMP on platelet secretion and aggregation might reside in their capacity to antagonize C-kinase activity.

INTRODUCTION

Platelets, like many other cells, have a bidirectional control system for transduction of an extracellular signal to the cell interior. Platelet activation by excitatory stimuli coincides with an increase in intracellular Ca²⁺ [1–4] that initiates the cellular response; inhibitory stimuli provoke an increase in cyclic AMP and inhibit the Ca²⁺-induced response [5–9]. The inhibitory effect was suggested to be due to a decrease in cellular free Ca²⁺ [3,10,11], either by a decreased mobilization [12] or an accelerated removal [13]. At the biochemical level, excitatory stimuli such as thrombin, collagen, platelet-activating factor, ADP and 5-hydroxytryptamine [14–18] induce rapid changes in inositol-lipid metabolism, most probably by the action of phospholipase C [Scheme 1, (1*)]. Diacylglycerol, a second messenger thus formed,

activates the C kinase that catalyses the phosphorylation of a 40 kDa protein [Scheme 1, (2)] [19]. It was suggested that the inhibitory stimuli inhibit phospholipase C [20,21] or diminish the re-synthesis of polyphosphoinositides that are substrates for the enzyme [9,22] [Scheme 1 (5)–(9)]. Both effects result in a decreased generation of second-messenger diacylglycerol and a concomitant decrease of C kinase activity.

Prostaglandins (i.e. PGE₁, PGD₂, PGI₂) and forskolin (FK) are commonly used inhibitory platelet stimuli [5,23–25]. They increase cellular cyclic AMP [5,23–25] and, as a consequence, several proteins become rapidly phosphorylated, most probably through the activation of a cyclic-AMP-dependent protein kinase [13,26–29]. The C kinase can be activated directly by 1-oleoyl-2-acetylgllycerol (OAG) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [30,31] [Scheme 1, (2)]. In the present paper we demonstrate that agents that are known to increase cellular cyclic AMP antagonize the activation of the C kinase.

EXPERIMENTAL

Materials

TPA, PGE₁ and phospholipids were obtained from Sigma (St. Louis, MO, U.S.A.). 5-Hydroxytryptamine (serotonin) came from Janssen Chimica (Beerse, Belgium) and forskolin from Calbiochem (La Jolla, CA, U.S.A.). Silica-gel-60 precoated plastic sheets were from E. Merck, Darmstadt, West Germany. [³²P]P_i, quin-2/AM and the cyclic AMP radioassay kit were obtained from Amersham International (Amersham, Bucks., U.K.).

Reference proteins for *M_r* determinations on SDS/polyacrylamide gels came from Bio-Rad (Richmond, CA, U.S.A.). OAG was kindly provided by Professor Dr. Y. Nishizuka (Kobe University, Kobe, Japan).

Scheme 1. The inositol phospholipid cycle

- (1) Phospholipase C; (2) C-kinase activation; (3) diacylglycerol kinase; (4) CTP:phosphatide cytidyltransferase; (5) CDP:1,2-diacylglycerol-inositol phosphatidyltransferase; (6) PI kinase; (7) PIP phosphomonoesterase; (8) PIP kinase; (9) PIP₂ phosphomonoesterase.

Abbreviations used: PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; OAG, 1-oleoyl-2-acetylgllycerol; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PG, prostaglandin; FK, forskolin; DMSO, dimethyl sulphoxide.

Preparation of ^{32}P -labelled human platelets

Human venous blood from healthy volunteers was collected in 0.2 vol. of anticoagulant buffer [1.3% (w/v) citric acid, 2.5% (w/v) sodium acetate and 2% (w/v) dextrose]. Platelets were prepared from platelet-rich plasma by repeated centrifugation and washing as described previously [32], and suspended in buffer (25 mM-Hepes, pH 7.6, 125 mM-NaCl, 2.7 mM-KCl, 5.6 mM-MgSO₄, 10 mM-glucose, 0.1 mM-EGTA) at 5×10^8 cells/ml. Unless otherwise stated, they were labelled with [^{32}P]P_i (approx. 250 $\mu\text{Ci}/\text{ml}$) at 37 °C for 70 min. At that time phosphatidic acid (PA) was labelled to near isotopic equilibrium [33,34]. Changes in the ^{32}P content of these phospholipids thus reflect their chemical changes.

Measurement of labelled lipids

Platelet samples (7.5×10^7 cells) were transferred rapidly into 4.5 ml of chloroform/methanol/0.75 M-HCl (5:10:2, by vol.). Two phases were obtained after the addition of 1.25 ml of chloroform and 1.2 ml of 0.1 M-HCl. After centrifugation (500 g, 10 min), the organic phase was washed with 2.5 ml of methanol/0.2 M-HCl (1:1, v/v) and re-centrifuged. The organic phase was evaporated under N₂, the residue dissolved in 75 μl of chloroform/methanol/water (75:25:2, by vol.) and applied to silica-gel-60-precoated plastic sheets. The sheets were eluted with chloroform/methanol/20% (v/v) methylamine (12:7:2, by vol.) for phospholipid analysis.

As described previously [18], ^{32}P -labelled phospholipids were located on the sheets after overnight autoradiography on Kodak X-Omat films; they were cut out and the radioactivity was quantified by liquid-scintillation spectrometry.

Measurement of ^{32}P -labelled proteins

Platelet samples (4×10^7 platelets) were quenched in a 5-fold-concentrated sample buffer [35] and incubated at 90 °C for 5 min. Proteins were separated by SDS/polyacrylamide-gel electrophoresis in a 12.5% -acrylamide gel by using the Laemmli [35] buffers. Gels were stained with Coomassie Brilliant Blue, dried and subjected to autoradiography. Areas carrying the proteins of interest were cut out and radioactivity was determined by liquid-scintillation counting.

The M_r of these proteins was estimated by using reference proteins.

Determination of cyclic AMP

For this, a radioimmunoassay kit was used. Trichloroacetic acid was added to the platelets to a final concentration of 10% (w/v). Protein precipitate was discarded after centrifugation (900 g, 10 min). The supernatant was collected, and trichloroacetic acid was extracted by three subsequent diethyl ether extractions. Further steps were performed exactly as described by the manufacturer.

Data calculation

At the beginning and the end of each experiment, duplicate platelet samples were taken in which the control ^{32}P incorporation in lipids and proteins was determined. [This control incorporation was always determined in the presence of the solvent(s) in which compounds used in the experiment were dissolved (0.1%

ethanol, 0.1% DMSO or 0.9% NaCl). Solvents alone had no influence on ^{32}P incorporation into either lipids or proteins.] Preliminary experiments showed us that, except for PA, PIP and PIP₂, labelling often increased slightly but near-linearly within the time course of an experiment (results not shown). The difference never exceeded 15%. For data calculation, the increase was accepted to be linear, and control ^{32}P incorporation at any time point of the experiment could be computed. The changes after addition of a compound were calculated as a percentage of the control ^{32}P incorporation (100%).

Within the different experiments, the control labelling also varied (at least partly due to blood-donor differences). These values were in the ranges 2000–5000 c.p.m. for PA, 35000–100000 c.p.m. for PI and PIP, 100000–230000 c.p.m. for PIP₂, 1000–2700 c.p.m. for 40 kDa protein and 350–1000 c.p.m. for 20 kDa protein. Regardless of the numerical difference in results obtained from the separate experiments, our method of computation allowed us to calculate the means of several experiments as shown in the graphical presentations.

RESULTS

The signal-transducing system of the platelet serotonin-S₂ receptor involves inositol-lipid metabolism [18,36]. On addition of 5-hydroxytryptamine (5 μM) to human platelets, [^{32}P]PA, [^{32}P]40 kDa protein and [^{32}P]20 kDa protein rapidly increase [36]. These three responses are easily measurable parameters of 5-hydroxytryptamine-induced phospholipase C, C kinase and myosin light-chain kinase respectively. Fig. 1 illustrates that PGE₁ and FK antagonize these three responses in a concentration-dependent manner. The 40 kDa-protein phosphorylation provoked by 5-hydroxytryptamine is completely inhibited; the 20 kDa protein phosphorylation decreases below control values at high PGE₁ and FK concentrations. FK as well as PGE₁ inhibited 40 kDa- and 20 kDa-protein phosphorylation at lower concentrations than for inhibition of PA formation. PA formation was only partially antagonized. If the inhibitory effect of PGE₁ and FK were solely due to their effect on phospholipase C [Scheme 1, (1*)] [20,21] or on the formation of its substrate(s) [Scheme 1 (5)–(9)] [9,22], one would expect the same concentration-dependency for inhibition of [^{32}P]PA formation and of 40 kDa protein [^{32}P]phosphorylation. Fig. 1, however, clearly shows that much higher concentrations are needed to block the former only partially than to affect protein phosphorylation.

We therefore investigated whether PGE₁ and FK counteract C kinase activity at a level beyond phospholipase C. For this investigation we used TPA and OAG, since these compounds directly stimulate C kinase [30,31]. When 40 kDa-protein phosphorylation is stimulated by TPA, the addition of PGE₁ or FK results in an immediate decrease in phosphorylation as compared with the control (Fig. 2).

In view of the higher inhibitory potency of PGE₁ as compared with FK, we used the former compound to increase platelet cyclic AMP in further experimental work. Table 1 shows that maximum concentrations of cyclic AMP were attained 5 s after addition of PGE₁ (1 μM) and that they remained elevated for at least 55 s.

Fig. 1. Concentration-dependent effect of PGE₁ and FK on 5-hydroxytryptamine-induced 40 kDa- and 20 kDa-protein phosphorylation and PA formation

Platelets prelabelled with [³²P]P_i were preincubated for 10 s with PGE₁, FK or solvent (ethanol, 0.01%). Then 5-hydroxytryptamine (5 μ M) was added. Samples were taken at 10–20 s for protein analyses and duplicate samples at 30 s for lipid analysis. ●, ■, ▲, PGE₁; ○, □, △, FK; ●, ○, 40 kDa protein; ■, □, 20 kDa protein; ▲, △, PA. Points represent the means of five separate experiments. The increase in ³²P-labelled 40 kDa protein, 20 kDa protein and PA owing to stimulation with 5-hydroxytryptamine was taken as 100%; '% stimulation' was calculated by analogy with the method described in ref. [18]. For the 40 kDa protein the increase in phosphorylation was $85.8 \pm 8.3\%$, for 20 kDa protein it was $109.6 \pm 11.2\%$, and for PA formation $187.5 \pm 13.3\%$ (all means $\pm$ S.E.M.; $n = 5$). PGE₁ and FK had no effect on [³²P]PA in unstimulated platelets [52].

Fig. 3 illustrates the potency of PGE₁ (1 μ M) to antagonize the 40 kDa protein phosphorylation induced by different concentrations of OAG. Maximum C-kinase activation was obtained at OAG concentrations above 25 μ M. PGE₁ decreased 40 kDa-protein phosphorylation at all OAG concentrations tested. At 3.2 μ M-OAG the activation was completely abolished by PGE₁. To evaluate the possibility that PGE₁ affects the metabolism of OAG, leading to lower OAG concentrations and a concomitant decrease in C-kinase activity, we replaced OAG by the phorbol ester TPA, which is not readily metabolized. Fig. 4 illustrates that TPA-induced 40 kDa-protein phosphorylation is also decreased on addition of PGE₁ (1 μ M), but less markedly so than in the experiment with OAG.

To gain further insight into the working mechanism, we investigated the effect of different concentrations of PGE₁ on OAG-stimulated [30,37] and basal 40 kDa- and 20 kDa-protein phosphorylation. We selected OAG

instead of TPA as C-kinase activator, since this synthetic diacylglycerol more closely resembles the natural co-factor of the enzyme.

Fig. 5 shows that direct stimulation of the C kinase by 5 μ M OAG is inhibited by PGE₁ in a concentration-dependent manner. The ³²P content of the 40 kDa protein, in the absence of OAG, remained unaltered. In contrast, the 20 kDa-protein phosphorylation decreased in a PGE₁-concentration-dependent way below the control value, as did the OAG-stimulated 20 kDa-protein [³²P]phosphorylation. Since on the one hand the activation of the protein kinase C was demonstrated to be a Ca²⁺-dependent process [19,38], and on the other hand increased cyclic AMP was suggested to decrease platelet intracellular Ca²⁺ [3,10,11], we investigated whether a cyclic-AMP-induced decrease in Ca²⁺ might explain the decrease in C-kinase activity that we observed. The loading of platelets with the membrane-permeant Ca²⁺ chelator quin 2/AM in the absence of

Fig. 2. PGE₁- and FK-induced decrease in 40 kDa-protein phosphorylation 30 s after stimulation with TPA

Platelets were prelabelled with [³²P]P_i. Samples were taken and TPA (5 nM) (in DMSO, 0.1% final concn.) was added at 0 s. After 30 s duplicate samples were taken and PGE₁ (1 μM) (Δ), FK (10 μM) (□) or solvent (0.1% ethanol) (○) was added. The stimulation was stopped as described in Fig. 1. Points represent the means of two experiments with duplicate samples. The difference in ³²P incorporation in the 40 kDa protein before and 30 s after addition of TPA was taken as 100%. For the two separate experiments ³²P incorporation increased by 33.2 and 39.4% during that time course.

Table 1. Changes in cyclic AMP on addition of PGE₁ (1 μM) to isolated platelets

Results represent means ± S.E.M. for four determinations.

Time (s)	Cyclic AMP (pmol/10 ⁸ platelets)
0	2.1 ± 0.4
5	36.8 ± 9.9
15	40.7 ± 12.5
60	35.0 ± 16.6

extracellular Ca²⁺ has previously been shown to decrease the intracellular free Ca²⁺ [39,40] and to impair severely excitatory stimulation of platelets [41–43]. Fig. 6 illustrates the effect of intracellular Ca²⁺ complexation by quin 2 on stimulation of human platelets with 5-hydroxytryptamine. In the presence of 50 μM-quin-2/AM (corresponds to 5–8 mM intracellular quin-2), the 5-hydroxytryptamine-induced PA formation and 40 kDa-protein phosphorylation were nearly completely abolished, indicating that the Ca²⁺ requirements for the activation of phospholipase C are no longer fulfilled. Fig. 7 illustrates the effect of intracellular Ca²⁺ complexation on direct activation of the protein kinase C. The OAG-induced 40 kDa-protein phosphorylation was decreased as compared with the control in the absence of quin 2; PGE₁ (1 μM) without quin-2/AM, however, nearly completely abolished 40 kDa-protein phosphoryl-

Fig. 3. Effect of PGE₁ on 40 kDa-protein phosphorylation after stimulation with different concentrations of OAG

PGE₁ (1 μM) (○) or its solvent (ethanol, final concn. 0.01%) (●) was added to platelets prelabelled with [³²P]P_i. After 10 s OAG (in DMSO, final concn. 1%) was added and the incubation proceeded for 35 s, after which it was stopped as described in Fig. 1. The control ³²P incorporation was taken as 100% PGE₁ itself had no effect on 40 kDa-protein phosphorylation (see also Fig. 6). Points represent means ± S.E.M. for three experiments with duplicate samples. Data were calculated as described in the Experimental section.

Fig. 4. Effect of PGE₁ on 40 kDa-protein phosphorylation after stimulation with different concentrations of TPA

PGE₁ (1 μM) (○) or its solvent (ethanol, final concn. 0.01%) (●) was added to platelets prelabelled with [³²P]P_i. After 10 s, TPA (in DMSO, final concn. < 0.1%) was added and the incubation proceeded for 45 s, after which it was stopped as described in Fig. 1. The control ³²P incorporation was taken as 100%. Points represent means ± S.E.M. for two experiments with duplicate samples. Data were calculated as described in the Experimental section.

Fig. 5. PGE₁-concentration-dependent effect on OAG-induced 40 kDa- and 20 kDa-protein phosphorylation

Platelets prelabelled with [³²P]P_i were preincubated for 10 s with PGE₁ (in ethanol, final concn. 0.1%). Then OAG (5 μM) (●, ■) or solvent (DMSO, 0.1%) (○, □) was added. After 45 s the incubation was stopped as described in the legend of Fig. 1. The control ³²P incorporation was taken as 100%. Changes in protein phosphorylation induced by OAG in the presence of PGE₁ were calculated by analogy with the method described in ref. [18]. ●, ○, 40 kDa protein. ■, □, 20 kDa protein. Without PGE₁, OAG-induced 40 kDa-protein phosphorylation was 289 ± 21% and 20 kDa-protein phosphorylation was 135 ± 5% (both ± S.E.M.; *n* = 7) of the control. Points represent the means ± S.E.M. for seven experiments with duplicate samples. Data were calculated as described in the Experimental section.

ation. These data illustrate that in platelets (i) receptor-activated PA formation (as a measure for phospholipase C) requires higher intracellular free Ca²⁺ concentrations than does 40 kDa-protein phosphorylation (as measured for protein kinase C activation) and (ii) the situation of decreased intracellular Ca²⁺ that we used had a smaller antagonizing effect on protein kinase C activity than did addition of PGE₁.

On addition of PGE₁ or FK to human platelets, the ³²P content of a 50 kDa protein, a 27 kDa protein or a 24 kDa protein markedly increased (results not shown). The ³²P content of none of these proteins was altered on stimulating platelets with 5-hydroxytryptamine, OAG or TPA (results not shown).

DISCUSSION

Agents that increase platelet cyclic AMP were suggested to inhibit the receptor-coupled polyphosphoinositide-specific phospholipase C [20,21] or to diminish the re-synthesis of its substrates [9,22]. Fig. 1 shows that such a mechanism is activated by PGE₁ and FK on activation of the 5-hydroxytryptamine S₂ receptor in platelets, since [³²P]phosphorylation of both 40 kDa protein and PA is decreased by these agents. The 5-hydroxytryptamine-induced increase in myosin light-chain phosphorylation (20 kDa protein) is also abolished

Fig. 6. Effect of intracellular Ca²⁺ complexation on 5-hydroxytryptamine-induced PA formation and 40 kDa-protein phosphorylation

Platelets prelabelled with [³²P]P_i were preincubated for 8 min with quin-2/AM (50 μM) (○, □) or its carrier (in DMSO, final concn. 0.1%) (●, ■) for 8 min. Samples were taken (zero time), and subsequently 5-hydroxytryptamine (5 μM) was added. Further sampling was performed at the indicated times. ●, ○, PA; ■, □, 40 kDa protein. Results represent means of duplicate samples from one experiment and are representative for five experiments.

Fig. 7. Effect of intracellular Ca²⁺ complexation and PGE₁ on OAG-induced protein kinase C activation

Platelets prelabelled with [³²P]P_i were preincubated for 8 min with quin-2/AM (50 μM) (▽) or for 10 s with PGE₁ (1 μM; ○) or their carriers (●). Samples were taken (zero time) and subsequently OAG (5 μM) was added. Further sampling was performed at the indicated times. Results represent means ± S.D. for four experiments with duplicate samples. The values at zero time are the means of the separate experiments; the values at later times are expressed as a percentage of the zero-time values.

by PGE₁ and FK. Myosin light-chain kinase is a Ca²⁺-calmodulin-dependent enzyme [44]. The effects of these compounds on the amount of ³²P-labelled 20 kDa protein are therefore consistent with their antagonistic effect on Ca²⁺ mobilization [4].

In the human platelet, 5-hydroxytryptamine induces shape changes and reversible aggregation without secretion [45]; the cellular free Ca²⁺ concentrations are raised [4]. Previous reports [4,46] showed that these phenomena were inhibited by PGE₁ and FK in the concentration range in which they inhibit 5-hydroxytryptamine-induced increases in 40 kDa- and 20 kDa-protein phosphorylation and PA formation (Fig. 1). More surprising, however, was our finding that higher concentrations on PGE₁ and FK were needed to block PA formation only partially than to affect protein phosphorylation (Fig. 1). Similar data were obtained when platelets were stimulated with thrombin in the presence of prostacyclin [9]. At high concentrations of the prostaglandin, aggregation was abolished, whereas the inositol phosphate release, which is a measure for phospholipase C or a preceding metabolic step, was not completely inhibited [9]. As an alternative mechanism for the only partial decrease in PA formation, an accelerated conversion of PA into PI [Scheme 1 (4) and (5)] can be postulated. Such cyclic-AMP-induced alterations were demonstrated in the platelet [47,48]. The high ³²P content of the PI pool in our experiments, however, would not allow us to detect a PA → PI conversion. This alternative mechanism does not explain the discrepancy between 40 kDa protein phosphorylation and PA formation.

Direct activation of the C kinase by OAG and TPA allowed us to study the effect of PGE₁ and FK at the level beyond phospholipase C. At the different concentrations of OAG and TPA tested, 40 kDa-protein phosphorylation was decreased by PGE₁ (Figs. 3 and 4). Its antagonistic effect, however, was more pronounced when OAG was used instead of TPA. A PGE₁-induced breakdown of OAG might contribute to this difference. The data obtained with TPA, however, show that PGE₁ can counteract the signal transduction of excitatory stimuli at the level of the C kinase. By analogy, Rink & Sanchez [49] found that agents that increase platelet cyclic AMP more potently antagonized the dense-granule ATP secretion induced by OAG than the release induced by TPA. In the same paper these authors show that the collagen-provoked ATP release in the presence of indomethacin is not inhibited by FK. More recently, Lapetina's group [50] illustrated that, in these conditions, protein kinase C is not activated, which explains why FK could have no inhibitory effect.

The experiment in which the PGE₁-concentration-dependence of this phenomenon is elaborated (Fig. 5) shows that the ³²P content of the 40 kDa protein in the unstimulated platelet remains unaffected; the stimulated C-kinase activation, however, was inhibited by PGE₁ in a dose-dependent way. The concentration of PGE₁ necessary to obtain half-maximal inhibition of OAG-induced 40 kDa-protein phosphorylation (approx. 22 nM; Fig. 5) was higher than that required to obtain half-maximal inhibition of 5-hydroxytryptamine-induced 40 kDa-protein phosphorylation (approx. 4.6 nM; Fig. 1). These data are in accordance with those on ATP secretion [49] that illustrated a higher inhibitory effect on thrombin-induced ATP secretion of agents that increase platelet cyclic AMP compared with that evoked by OAG.

It is tempting to conclude from our data that the decrease in ³²P content is not caused by a direct effect on either the kinase or the phosphatase activity, since that would also affect the 40 kDa-protein phosphorylation in unstimulated platelets, as can be seen for 20 kDa-protein phosphorylation. However, our knowledge about the function of 40 kDa protein is too poor to exclude that kinase/phosphatase systems other than that involved in protein kinase C activation contribute to the ³²P content of the 40 kDa protein in resting platelets. A direct effect on either the protein kinase C or a phosphatase should therefore still be envisaged. The effect of PGE₁ on 20 kDa-protein phosphorylation is clearly different. The basal as well as the OAG-stimulated phosphorylation decrease below the control value. A direct effect on the phosphorylation/dephosphorylation processes is hence apparent. Since the myosin light-chain kinase is a Ca²⁺-calmodulin-dependent enzyme [44] and is probably responsible for basal extents of phosphorylation, decreased intracellular Ca²⁺ [3,4,10,13,26] might explain these observations.

By analogy with the myosin light-chain kinase, the intracellular Ca²⁺ concentrations might determine the protein kinase C activity, as was found by other investigators [19,38]. The experiment described in Figs. 6 and 7, in which we used quin 2 to decrease the intracellular Ca²⁺ concentration, demonstrated that, under conditions in which phospholipase C activity was nearly completely abolished (Fig. 6), protein kinase C activity retained approx. 70% of its phosphorylating capacity (Fig. 7). In case the antagonistic effect on 40 kDa-protein phosphorylation of compounds that increase platelet cyclic AMP is due to a decrease in intracellular free Ca²⁺ concentration, these compounds would antagonize phospholipase C activity more potently than protein kinase C activity. The experiment described in Fig. 1, on the contrary, illustrates that higher concentrations of either PGE₁ or FK are required to inhibit PA formation activity only partly than to decrease 40 kDa protein phosphorylation.

We can therefore conclude that a PGE₁- or FK-induced decrease in platelet intracellular free Ca²⁺ plays only a minor role in the antagonistic effect of these compounds on protein kinase C activity.

It is unknown to what extent compounds that increase cyclic AMP can reverse C-kinase activation by a physiological agonist. On stimulation with 5-hydroxytryptamine (5 μM), the increase in 40 kDa-protein [³²P]phosphorylation was approx. 85%, which is similar to that with 30 nM-TPA (Fig. 4) and less than that by 3 μM-OAG (Fig. 3). At these concentrations of TPA and OAG, C-kinase activation was abolished by PGE₁ (Figs. 3 and 4). It is therefore highly suggestive that the discrepancy that we found between the PGE₁- or FK-induced decrease in 40 kDa-protein phosphorylation on the one hand and PA formation on the other (Fig. 1) will be due to the antagonistic effect that we found to occur at the level of the C kinase. The finding that one pathway of the bifurcating excitatory signal-transduction system in platelets is antagonized by compounds that increase cyclic AMP might be the key event in the anti-aggregatory effect of these compounds.

To our knowledge, our data provide the first biochemical evidence that compounds known to increase platelet cyclic AMP can have an inhibitory effect in the excitatory signal transduction at a site beyond phospho-

lipase C at the level of the C kinase. It should be mentioned that an inhibitory site for cyclic AMP beyond phospholipase C has been suggested, but based on physiological evidence, from a study on the effect of increased cyclic AMP on platelet secretion [51]. The precise nature of this mechanism, however, remains to be elucidated.

Whether or not cyclic AMP is causally related to the effects seen in our study with PGE₁ and FK cannot be decided from the present work. These compounds might indeed affect other processes besides an increase in cyclic AMP.

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