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Collagen-induced platelet activation mainly involves the protein kinase C pathway

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This study analyses early biochemical events in collagen-induced platelet activation. An early metabolic event occurring during the lag phase was the activation of PtdIns(4,5)P_2-specific phospholipase C. Phosphatidic acid (PtdOH) formation, phosphorylation of P43 and P20, thromboxane B_2 (TXB_2) synthesis and platelet secretion began after the lag phase, and were similarly time-dependent, except for TXB_2 synthesis, which was delayed. Collagen induced extensive P43 phosphorylation, whereas P20 phosphorylation was weak and always lower than with thrombin. The dose–response curves of P43 phosphorylation and granule secretion were similar, and both reached a peak at 7.5 μg of collagen/ml, a dose which induced half-maximal PtdOH and TXB_2 formation. Sphingosine, assumed to inhibit protein kinase C, inhibited P43 phosphorylation and secretion in parallel. However, sphingosine was not specific for protein kinase C, since a 15 μM concentration, which did not inhibit P43 phosphorylation, blocked TXB_2 synthesis by 50%. Sphingosine did not affect PtdOH formation at all, even at 100 μM, suggesting that collagen itself induced this PtdOH formation, independently of TXB_2 generation. The absence of external Ca^{2+} allowed the cleavage of polyphosphoinositides and the accumulation of InsP_3 to occur, but impaired P43 phosphorylation, PtdOH and TXB_2 formation, and secretion; these were only restored by adding 0.11 μM-Ca^{2+}. In conclusion, stimulation of platelet membrane receptors for collagen initiates a PtdInsP_2-specific phospholipase C activation, which is independent of external Ca^{2+}, and might be the immediate receptor-linked response. A Ca^{2+} influx is indispensable to the triggering of subsequent platelet responses. This stimulation predominantly involves the protein kinase C pathway associated with secretion, and appears not to be mediated by TXB_2, at least during its initial stage.

INTRODUCTION

Primary haemostasis and thrombosis result from the adhesion of circulating platelets to sub-endothelial collagen. Secondarily to adhesion, the stimulation of membrane receptors alters platelet metabolism and leads to secretion and aggregation. The identification of collagen-specific receptor(s) is still under investigation [1–4], and the mechanism of the signal transduction that follows receptor stimulation is not clear. In many cell types, inositol lipid metabolism is considered an important factor in initiating cell responses to agonists; in particular, the PtdInsP_2 hydrolysis by phospholipase C leads to the formation of two second messengers: one is 1,2-diacylglycerol (DAG), which activate the protein kinase C sensitive to Ca^{2+} and phospholipids, in order to phosphorylate a 43 kDa protein (P43); the other messenger is inositol 1,4,5-trisphosphate (InsP_3), which is one of the factors involved in mobilizing Ca^{2+} from the dense tubular system, and therefore permits the activation of a Ca^{2+}–calmodulin-dependent myosin light-chain kinase to phosphorylate the myosin light-chain protein (P20) (for reviews see [5–7]). Collagen allows the formation of InsP_3, but, unlike thrombin, it only mobilizes Ca^{2+} weakly [8–11]. In the present paper, we analysed the early biochemical events induced by collagen, including polyphosphoinositide breakdown, the formation of phosphatidic acid (PtdOH), the phosphorylation of P43 and P20, and thromboxane B_2 (TXB_2) synthesis, which might involve different pathways of activation. These events were studied concomitantly with platelet secretion and aggregation. Their dependence on extracellular Ca^{2+} was also examined, and the importance of the protein kinase C pathway in the collagen-induced platelet activation was investigated by studying the effect of sphingosine, assumed to inhibit protein kinase C activity [12].

EXPERIMENTAL

Materials

5-Hydroxy[^{14}C]tryptamine ([^{14}C]5HT) (18 GBq) and [^{32}P]P_i (carrier free; 37 MBq/ml) were purchased from CEA-ORIS (Saclay, France). Metrizamide was from Nyegaard (Oslo, Norway). Calf skin type I collagen (Stago, Asniere, France) was used in this study.

Radiolabelled platelet preparation

Human blood, collected from healthy volunteers who had not taken aspirin during the previous 10 days, was anticoagulated with $\frac{1}{6}$ vol. of ACD-C (13 mM-citric acid, 12.4 mM-sodium citrate, 11 mM-glucose). Platelet-rich plasma was obtained by centrifugation of whole blood at 150 *g* for 15 min. It was incubated for 90 min at 37 °C with either [^{14}C]5HT (0.6 μM) for secretion studies or with [^{32}P]P_i (37 MBq/ml) for studies of phospholipid metabolism and protein phosphorylation. Radiolabelled platelets were isolated on metrizamide gradients as previously described [13] and finally resuspended at 4×10^8 cells/ml in a buffer containing 10 mM-Hepes, 0.15 M-NaCl, 3 mM-KCl, 0.5 mM-$MgCl_2,6H_2O$, 5 mM-$NaHCO_3$ and 10 mM-glucose, pH 7.4.

Abbreviations used: InsP_3, inositol 1,4,5-trisphosphate; PtdOH, phosphatidic acid; DAG, diacylglycerol; β-TG, β-thromboglobulin; TXB_2, thromboxane B_2; 5HT, 5-hydroxytryptamine.

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Platelet aggregation

Platelets were divided into 0.4 ml batches. All samples were prewarmed at 37 °C in an aggregometer cuvette, and different concentrations of collagen were stirred into them. Aggregation was continuously monitored and evaluated turbidimetrically. Results were expressed in terms of increases in light transmission (percentage), the platelet suspension and suspending buffer being used as references of 0 and 100% transmission respectively. At the appropriate times, reactions were stopped and the following studies performed.

Determination of platelet release

^{14}C-labelled platelets were transferred at 4 °C into tubes containing 0.1 ml of 0.1 M-EDTA and centrifuged in a Beckman Microfuge. The secretion of 5HT was evaluated by liquid-scintillation counting of the ^{14}C radioactivity in 0.1 ml of the supernatant, and secretion of β-thromboglobulin (β-TG) was determined in the same supernatant by a commercial immunoassay kit (Abbot, France). [^{14}C]5HT and β-TG secretions were calculated as the percentage of total [^{14}C]5HT radioactivity and total β-TG content in unstimulated platelets.

InsP_3 formation

Platelets were transferred at 4 °C into tubes containing an equal volume of 15% (w/v) trichloroacetic acid. After sedimentation of proteins by centrifugation at 2000 *g* for 15 min at 4°C, supernatants were extracted and titrated to pH 7.5 with $NaHCO_3$. InsP_3 formation was evaluated in these supernatants by using a commercial radioassay kit (Amersham International, Amersham, Bucks., U.K.).

Phosphoinositide metabolism

^{32}P-labelled platelets were transferred into glass tubes containing 3.75 vol. of chloroform/methanol/12 M-HCl/0.1 M-EDTA (20:40:1:2, by vol.) at 4 °C. The mixture was separated into two phases by adding 1.25 vol. each of chloroform and water. Phase separation was completed by centrifugation for 10 min at 200 *g*. This method allows analysis of proteins and lipids in the same platelet sample, because the ^{32}P-labelled proteins concentrate at the interphase and the extracted lipids are recovered in the lower chloroform phase. [^{32}P]Phospholipids were separated by one-dimensional t.l.c. on silica plates as previously described [14]. ^{32}P-labelled PtdIns(4,5)P_2, PtdIns4P, PtdIns and PtdOH were detected by autoradiography, recovered by scraping the identified spots, and the radioactivity was counted.

Protein phosphorylations

The ^{32}P-labelled proteins recovered at the interphase were solubilized in buffer containing 2% (w/v) SDS, 20% (v/v) glycerol, 0.01% Bromophenol Blue and 0.625 M-Tris/HCl, pH 10. They were analysed by SDS/PAGE under reducing conditions, with 13% (w/v) acrylamide in the resolving gel and 5% acrylamide in the stacking gel. After staining with Coomassie Blue, gels were exposed to Kodak XAR film. Autoradiograms were scanned with an LKB Ultroscan instrument, and the radioactivity of each phosphorylated protein band was evaluated by densitometry.

Determination of TXB_2 synthesis

This was done by enzyme immunoassay on a sample of ^{32}P-labelled platelets in the organic mixture before phase separation, by using anti-TXB_2 antibodies labelled with acetylcholinesterase [15].

Calculation of external free Ca^{2+}

The external free Ca^{2+} was determined by the method of Fabiato & Fabiato [16], based on calculation of free Ca^{2+} in the presence of EGTA and taking into account the composition of the platelet resuspension buffer [16]. The external free Ca^{2+} was thus adjusted with solutions containing 0.5 mM-EGTA and different amounts of free Ca^{2+}.

RESULTS

Phospholipid metabolism

Phosphoinositide turnover (Fig. 1*a*). The onset of aggregation in human platelets exposed to collagen was preceded by a 40 s lag phase. The addition of collagen initiated a rapid decrease in endogenous PtdInsP_2, since ^{32}P-labelled PtdInsP_2 decreased significantly by 29% at 30 s after collagen addition. This loss of PtdInsP_2 was maintained during the shape change. Rapid resynthesis was then initiated, and raised the PtdInsP_2 level to 130% of that of the unstimulated platelet controls at the end of aggregation, 120 s after collagen addition. A transient delayed decrease in PtdIns$P$ was also observed, 10–20 s after the fall in PtdInsP_2 (Fig. 1*a*). No change in the ^{32}P radioactivity associated with PtdIns was ever detected.

Formation of PtdOH (Fig. 1*b*). A significant detectable increase in PtdOH accumulation (1.3±0.05% of basal level) was noted in all experiments, just before the onset of aggregation, 40 s after collagen addition. Thereafter PtdOH formation increased steadily, until it reached its maximum about 90 s after collagen addition.

Time-dependence studies (Fig. 2)

The time course of P43 and P20 phosphorylation given in Fig. 2(*a*) shows that phosphorylation of P43 occurred within 50 s of the addition of collagen, and thereafter progressively increased, reaching its half-maximum at 65 s, and its maximum at 2 min. The time course of P43 phosphorylation was parallel to that of platelet secretion (Fig. 2*b*). A weak phosphorylation of P20 was also observed as a function of time, but was always lower than that found previously in the presence of thrombin [17].

The secretion of both 5HT from dense granules and β-TG from α granules occurred concomitantly with aggregation (Fig. 2*b*). Half-maximal secretion was reached at 60 and 65 s respectively, and at 65 s for aggregation. Maximal 5HT and β-TG secretion was 27% and 43% of the total granule content respectively, and occurred 120 s after addition of 7.5 μg of collagen/ml. Whereas collagen induced secretion from dense and α granules, no *N*-acetylglucosamine activity was released from lysosomes (results not shown).

TXB_2 synthesis began 50 s after collagen addition, concomitantly with the phosphorylation of P43 and granule secretion (Fig. 2*c*). However, the time-course curve for TXB_2 synthesis shifted to the right compared with the curve for other platelet responses, since TXB_2 accumulation was slower, with a half-maximum value at 90 s compared with 60, 65 and 65 s for PtdOH formation, P43 phosphorylation and granule secretion respectively.

Dose-dependence of platelet responses

Maximal platelet aggregation was reached at the relatively low collagen concentration of 7.5 μg/ml, and 5HT and β-TG secretion rose with aggregation (Fig. 3). Thus, for all three, half-maximal responses were obtained with about 2.5 μg of collagen/ml. Secretion of 5HT and β-TG reached maxima of 33% and 52% of the total platelet content respectively, at 7.5 μg of collagen/ml.

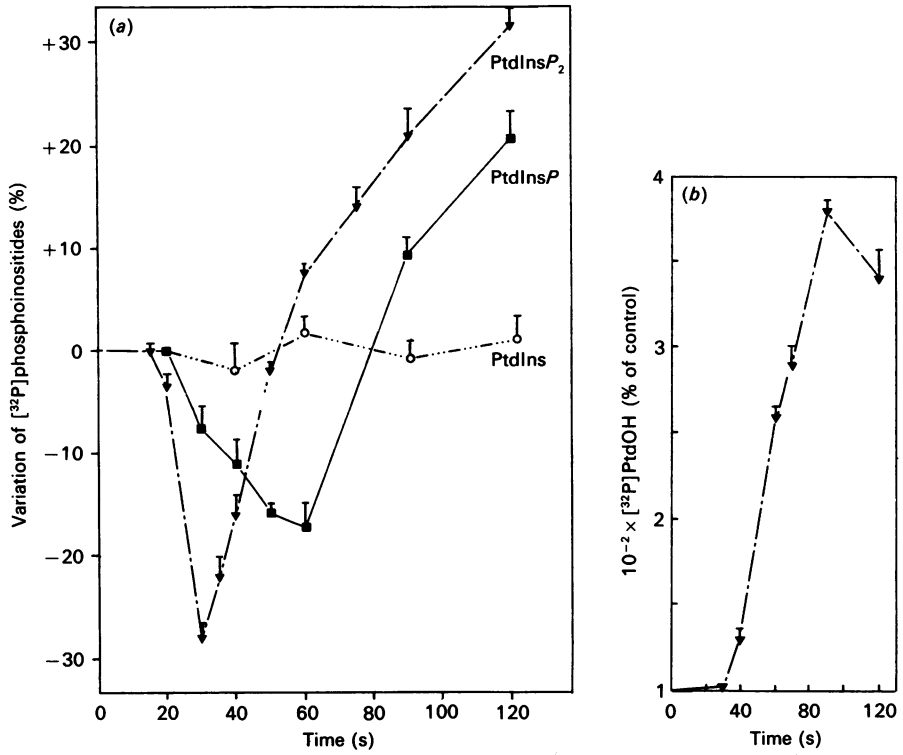

Fig. 1. Time course of [^{32}P]phosphoinositide variations and PtdOH formation in platelets exposed to collagen

^{32}P-labelled platelets were incubated with 7.5 μg of collagen/ml. At the indicated times, PtdInsP_2, PtdIns$P$, PtdIns and PtdOH were extracted as described in the Experimental section. (*a*) [^{32}P]Phosphoinositide variations: changes in ^{32}P radioactivity were calculated as percentage variations versus the control value (0%), which represented the radioactivity of [^{32}P]-PtdInsP_2, -PtdIns$P$ or -PtdIns in unstimulated platelets for each incubation period. (*b*) PtdOH formation: results are expressed as percentages of control values for unstimulated platelets. Results are means ± S.E.M. of five experiments.

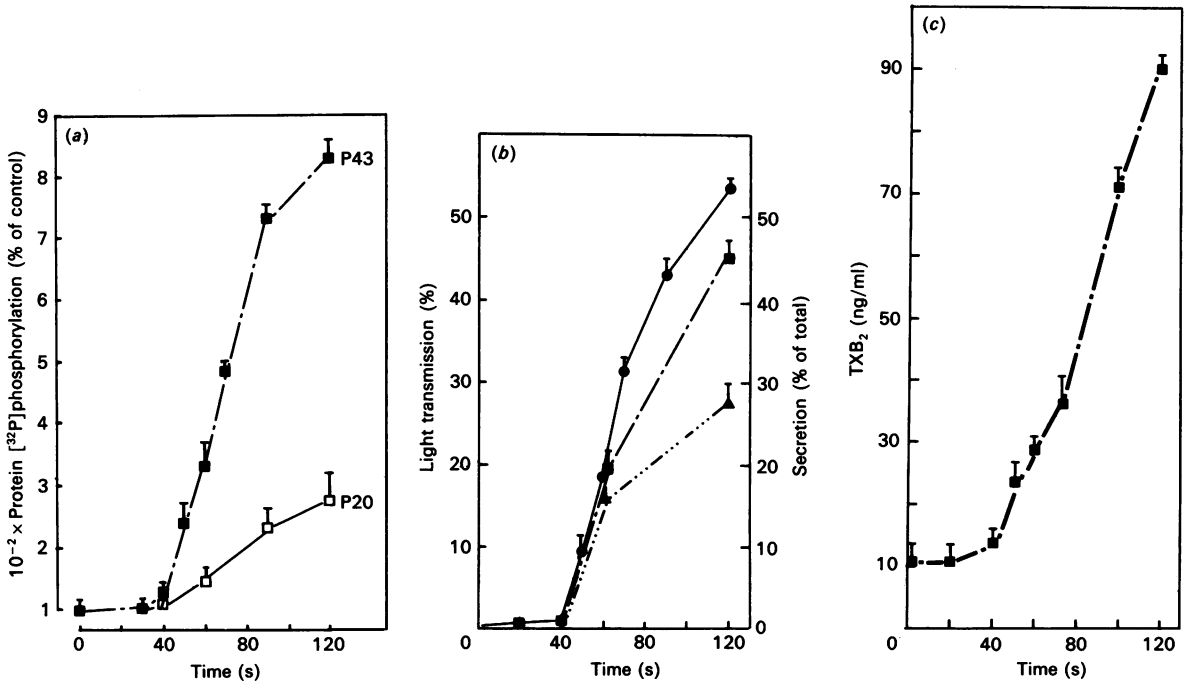

Fig. 2. Time course of protein phosphorylations, platelet secretion and TXB_2 synthesis

^{14}C- or ^{32}P-labelled platelets were activated in an aggregometer tube at 37 °C with 7.5 μg of collagen/ml. The incubations were stopped at the indicated times and analysed as described in the Experimental section. (*a*) Time course of protein phosphorylations: the radioactivity of the P43 (■) and P20 (□) proteins was evaluated by densitometry with an LKB scanner and expressed as percentage of control values for unstimulated platelets. (*b*) Time course of secretion and aggregation: secretion was measured as releases of 5HT (▲) and β-TG (■). Results are expressed as percentages of total granule content, from which the basal secretion of unstimulated stirred platelets was subtracted. The extent of aggregation (⊙) was expressed as the percentage of light transmission. (*c*) Time course of TXB_2 synthesis: results are expressed as ng/10^9 platelets. Results are means ± S.E.M. of five experiments.

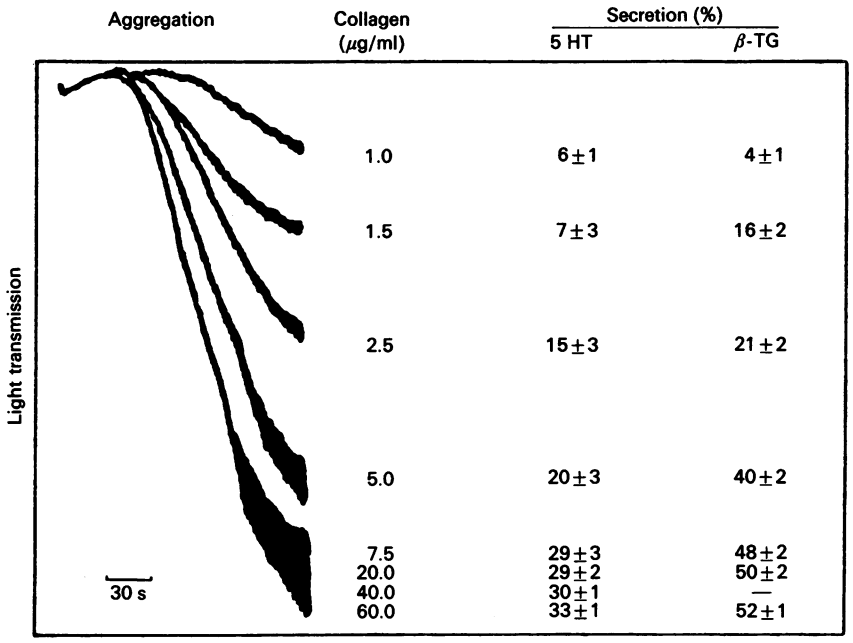

Fig. 3. Dependence of aggregation and secretion of 5HT and β-TG on the dose of collagen

Platelets were activated with increasing concentrations of collagen for 150 s at 37 °C. Secretion is expressed as the percentage of the total 5HT and β-TG. Results are means ± S.E.M. of five experiments.

Comparison of the collagen dose-dependence of P43 phosphorylation, PtdOH formation and TXB_2 synthesis is shown in Fig. 4. The dose–response curve for P43 phosphorylation showed that 2.5 μg of collagen/ml was sufficient for 50% phosphorylation of this protein. A dose of 7.5 μg of collagen/ml induced a full phosphorylation of P43 and complete secretion. However, this dose was necessary to reach 50% formation of PtdOH, and only 20% synthesis of TXB_2. The collagen concentrations necessary for maximal synthesis of PtdOH and TXB_2 were 4–5 times higher (40 μg/ml) than the 7.5 μg/ml necessary for complete phosphorylation of P43.

These dose-dependence experiments therefore provided further evidence for the parallelism between P43 phosphorylation and platelet secretion.

Role of external Ca^{2+}

When investigating the role of external Ca^{2+} in the regulation of platelet activation, we began by studying the dependence on external Ca^{2+} of inositol lipid hydrolysis. The platelet-suspending medium contains a residual contaminating Ca^{2+} concentration which may reach up to 50 μM without the addition of external Ca^{2+}. The absence of any external Ca^{2+} was therefore ensured by adding EGTA. In the presence of 0.5 mM-EGTA, collagen still induced the phosphodiesteratic cleavage of polyphosphoinositides. Fig. 5 illustrates the rapid accumulation of $InsP_3$, which reached its maximum (22.4 ± 1.2 pmol/ml) at the end of the shape change, 40 s after collagen addition. Thereafter the level of $InsP_3$ decreased, but remained higher than that in unstimulated platelets. In the presence of EGTA, a large transient increase in $InsP_3$ (up to 18 ± 1.3 pmol/ml) continued to be observed, and EGTA had only a little effect on the amount of $InsP_3$ accumulated. The time course and extent of the decrease in $PtdInsP_2$ were also not affected by EGTA. However, its presence did affect their resynthesis, which never exceeded the levels observed in unstimulated platelets.

As shown in Fig. 6, the absence of external Ca^{2+} completely prevented P43 phosphorylation, PtdOH formation, 5HT and β-TG secretion, TXB_2 synthesis and platelet aggregation. Fig. 6 clearly indicates that the addition of 0.1 μM exogenous Ca^{2+} was necessary to trigger platelet responses induced by collagen and to restore them by 80–90%.

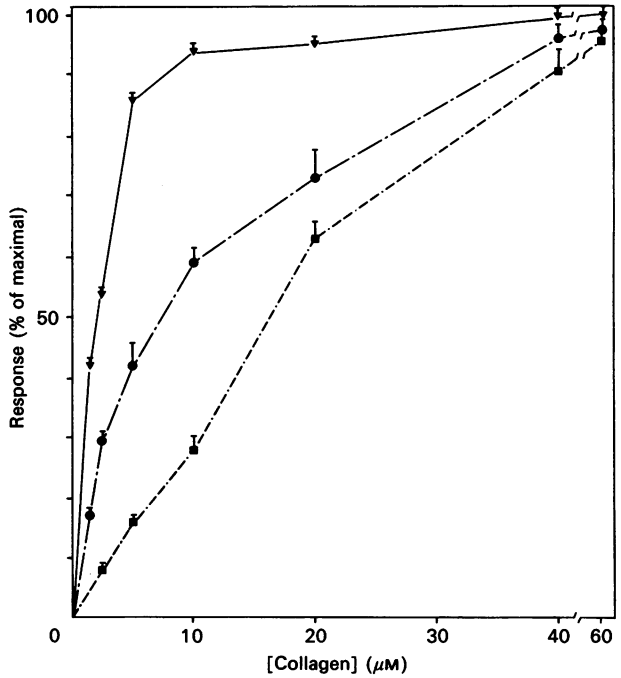

Fig. 4. Dependence of P43 phosphorylation (▼), formation of PtdOH (●) and TXB_2 synthesis (■) on collagen concentration

^{32}P-labelled platelets were incubated with different concentrations of collagen at 37 °C for 150 s. Results are expressed as percentage of the maximum response (100%) induced by 80 μg of collagen/ml. Maximal values were 360 ng/10^9 platelets for TXB_2, 13% of the phosphorylated proteins for P43, and 5.7-fold the basal level of unstimulated platelets for PtdOH.

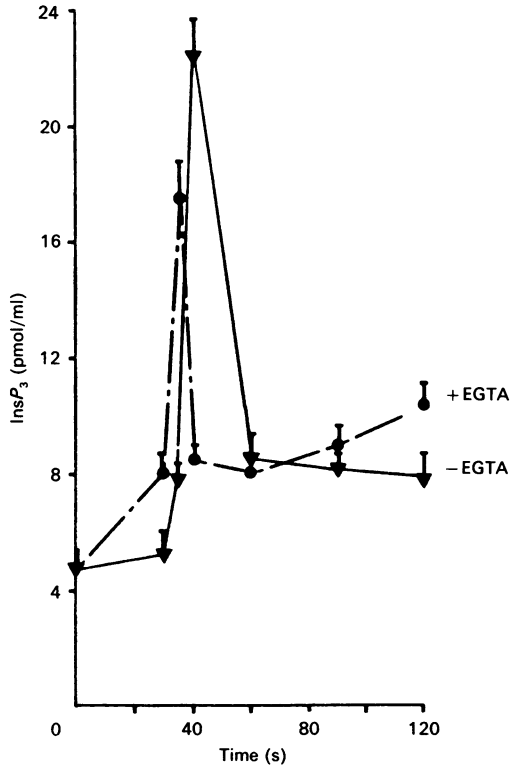

Fig. 5. Effect of EGTA on the time course of $InsP_3$ formation and $PtdInsP_2$ metabolism in platelets exposed to collagen

EGTA (0.5 mM) was preincubated with platelets (5×10^8/ml) at 37 °C for 15 s before the addition of collagen. At the indicated times, $InsP_3$ formation was determined as described in the Experimental section. Results are expressed in pmol/ml. Changes in [^{32}P]$PtdInsP_2$ radioactivity were calculated as percentage variations versus the control value (0%) representing the radioactivity of [^{32}P]$PtdInsP_2$ in unstimulated platelets. Results are means ± S.E.M. of three experiments.

Time (s)	Variations in percentage of $PtdInsP_2$	
	−EGTA	+EGTA
20	−3 ±2	−6 ±1
30	−28 ±1	−14 ±1
40	−16 ±2	−20 ±2
60	+7 ±1	−16 ±1
75	+14 ±2	−14 ±2
90	+21 ±2	−1 ±1
120	+32 ±2	+1 ±1

Effect of sphingosine (Fig. 7)

We showed previously that collagen induced marked phosphorylation of P43 but only weak phosphorylation of P20 compared with that elicited by thrombin [17]. The effect of sphingosine, a potent inhibitor of protein kinase C activity [12], was therefore investigated, in order to ascertain whether protein kinase C activation and the subsequent P43 phosphorylation are essential for collagen-induced platelet activation.

Sphingosine decreased P43 phosphorylation by 50% at a concentration of 50 μM, and completely inhibited it at 100 μM. Sphingosine also inhibited secretion of 5HT and β-TG with similar dose-dependence. However, sphingosine did not only seem to inhibit protein kinase C activity; it also inhibited TXB_2 synthesis, within a lower range of concentrations than for P43 phosphorylation. Thus, at 15 μM-sphingosine, inhibition of TXB_2 synthesis had already reached 50%, whereas phosphorylation of P43 and platelet secretion were still normal; 25 μM-sphingosine was the minimal dose required to inhibit P43 phosphorylation significantly by 15%. At this dose, TXB_2 synthesis was inhibited by 70%. It was noteworthy that PtdOH formation was not inhibited at all, even at 100 μM, and therefore continued to occur in the absence of TXB_2 synthesis and P43 phosphorylation.

DISCUSSION

Collagen is a potent platelet activator which induces metabolic and physiological events such as stimulation of the phosphatidylinositol cycle, protein phosphorylations and granule-content secretion [18–20]. The present results provide further insight into the mechanism initiating collagen-induced platelet activation. Collagen-induced platelet aggregation is characterized by a lag phase, at the end of which the release of granule contents starts. This only concerned the contents of α granules and dense bodies, since no release of *N*-acetylglucosamine activity from lysosomes was observed. Before the end of the lag phase, an early metabolic event was the activation of a phospholipase C specific for $PtdInsP_2$, leading to the formation of the second messengers DAG and $InsP_3$. DAG activates protein kinase C, which phosphorylates P43, and is also implicated in two main metabolic routes: it is phosphorylated by a DAG kinase to phosphatidic acid, and through a DAG lipase it can generate arachidonic acid, thus contributing to the synthesis of TXB_2. Our results showed that, at least within the limits of our methodology, all these events occur simultaneously after the lag phase. $InsP_3$ helps to mobilize Ca^{2+} from the dense tubular system [21–23], and Ca^{2+} permits the activation of the calmodulin-dependent myosin light-chain kinase to phosphorylate P20. With collagen, Ca^{2+} mobilization is known to be low, in contrast with the more extensive mobilization caused by thrombin [8–11]. This might explain the weak P20 phosphorylation that we detected with collagen and which remained consistently lower than with thrombin [17]. In contrast, the collagen-induced phosphorylation of P43 by protein kinase C was marked, and its extent was comparable with that observed with thrombin [17]. The mechanism of collagen-induced activation therefore seems to mediate preferentially protein kinase C activity and the subsequent phosphorylation of P43. This does not favour the concept that $InsP_3$ and DAG pathways act synergistically to produce a full physiological platelet response [24,25].

The present study showed, in agreement with others [26–28], that external Ca^{2+} was not required for the phosphodiesteratic cleavage of polyphosphoinositides. The decrease in [^{32}P]$PtdInsP_2$ could be due to a decrease in $PtdInsP_2$ dephosphorylation, an inhibition of PtdIns*P* phosphorylation, an activation of $PtdInsP_2$ hydrolysis, or a combination of these reactions. The occurrence of $InsP_3$ accumulation in the presence of EGTA suggests that the decrease in [^{32}P]$PtdInsP_2$ in response to collagen is due to the phospholipase C activity, although we cannot exclude that it could be also due to some of the other reactions. This is also consistent with the concept that activation of $PtdInsP_2$-specific phospholipase C is independent of external Ca^{2+}. The phospholipase C-catalysed hydrolysis of $PtdInsP_2$ induced by collagen may therefore constitute the immediate receptor-linked response of platelets to collagen.

However, the occurrence of $PtdInsP_2$ hydrolysis was not sufficient to initiate activation in the absence of external Ca^{2+}. The results of this study underline the importance of a Ca^{2+} influx in initiating the biochemical processes that follow platelet stimulation by collagen. The existence of such a cation translocation has previously been suggested [28,29]. Thrombin was

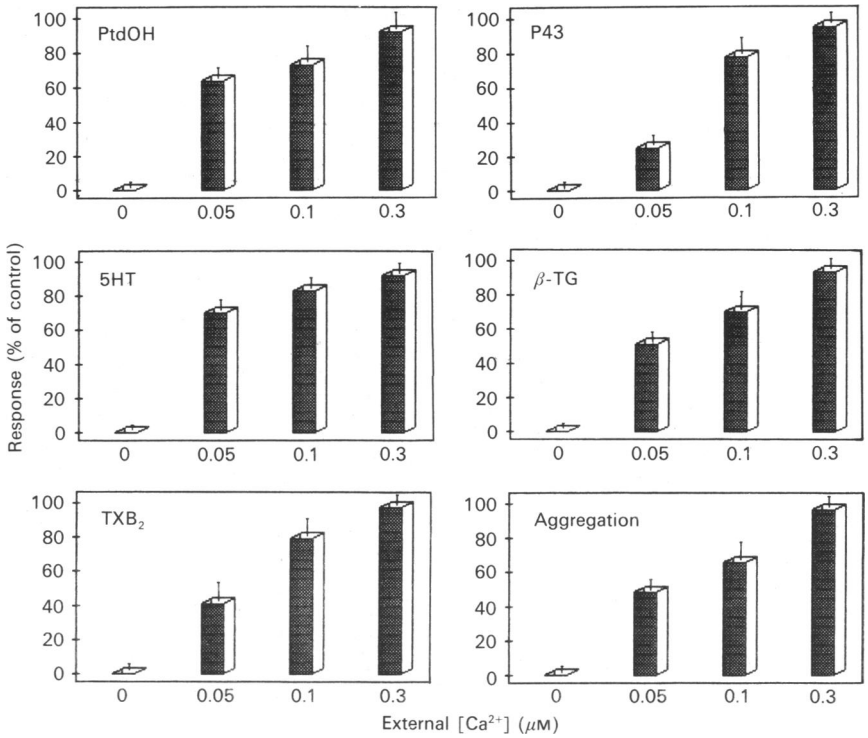

Fig. 6. Ca^{2+}-sensitivity of the different platelet responses

Platelets were stirred with 0.5 mM-EGTA (0 mM Ca^{2+}) or increasing concentrations of Ca^{2+} 15 s before the addition of collagen. The indicated Ca^{2+} concentrations are the exact concentrations of free Ca^{2+} in the suspending medium. They were calculated by the method of Fabiato & Fabiato [16], as described in the Experimental section. Results are expressed as percentages of the control values obtained in the absence of EGTA and of external Ca^{2+}.

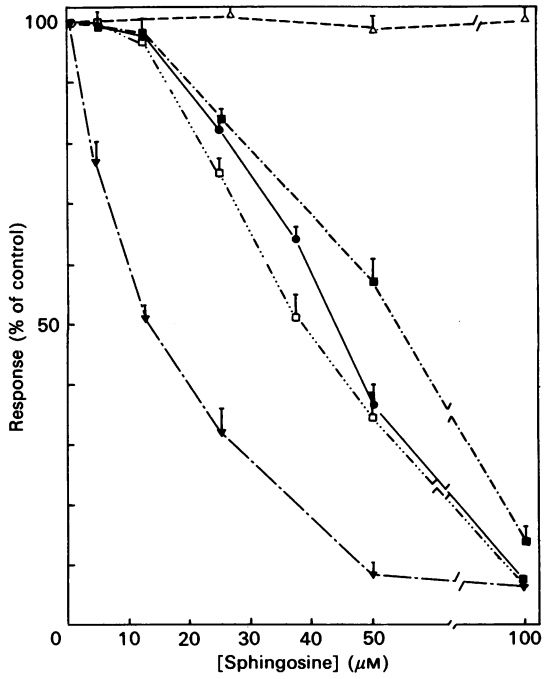

Fig. 7. Effect of sphingosine: concentration-dependence of P43 phosphorylation, secretion of 5HT and β-TG, and TXB_2 synthesis

Platelets were preincubated with various concentrations of sphingosine at 37 °C for 1 min before the addition of collagen. Results were expressed as the percentage of the maximal response (100%) corresponding to the control values for untreated platelets. Key: ▼, TXB_2; □, 5HT; ●, β-TG; ■, P43; △, PtdOH.

also able to stimulate the generation of $InsP_3$ in the presence of EGTA, but, unlike what occurred here with collagen, protein phosphorylation, PtdOH formation and platelet secretion were not inhibited [30]. However, with thrombin, the absence of external Ca^{2+} might have been overcome by its ability to cause extensive mobilization of Ca^{2+} from internal storage organelles [8,11].

From the present results, one of the essential roles of extracellular Ca^{2+} seems to be the activation of protein kinase C, whose activity is DAG- and Ca^{2+}-dependent [31]. In this connection, at least three forms of protein kinase C, with different Ca^{2+} and DAG requirements, have been described [32]. In our study, the greatest effect of collagen on P43 phosphorylation was observed with a free Ca^{2+} concentration of 0.1 μM, which was too low to raise the level of cytosolic Ca^{2+}. This agrees with the fact that protein kinase C activity is independent of a rise in cytosolic Ca^{2+}, and is fully activated by DAG at a Ca^{2+} concentration of 0.1 μM [7,33]. Furthermore, DAG is thought to convert primed protein kinase C into its activated Ca^{2+}-sensitive form, associated with the membrane. In this form, the protein kinase C activity has been shown to be regulated by the rate of Ca^{2+} influx [34,35]. However, the question also arises of whether the amount of DAG produced by $PtdInsP_2$ hydrolysis in the present study was sufficient, since no PtdIns hydrolysis was observed, or whether an additional Ca^{2+}-dependent source of DAG is required for full activation of protein kinase C. In this respect, it has been suggested previously that, in cells other than platelets, part of the increase in DAG observed during stimulation was probably derived from phospholipids other than phosphoinositides [36].

Our results suggest that P43 phosphorylation was one of the main events that follows collagen stimulation and that it closely

correlated with the release reaction. In this connection, studies with the protein kinase C inhibitor sphingosine are also of particular significance, since they suggest that P43 phosphorylation has a key role in collagen-induced platelet secretion. However, sphingosine does not seem to be as specific for protein kinase C as was originally believed. Thus, in this study, it inhibited TXB_2 synthesis at lower concentrations than for P43 phosphorylation and platelet secretion.

Many authors have suggested that platelet responses to collagen are dependent on thromboxane generation, since rises in intracellular [Ca^{2+}], specific protein phosphorylation and secretion were markedly affected by cyclo-oxygenase blockade [37,38]. Thromboxanes are known to interact with specific platelet membrane receptors that are coupled to the activation of the phosphoinositide cycle and to the increase in intracellular [Ca^{2+}] [39,40]. Thus the initial responses to collagen were thought to be mediated by thromboxanes. However, the question arises as to how the free arachidonic acid necessary for TXB_2 synthesis is released during the initial platelet–collagen interaction. An interesting result of our study was that, in sphingosine-treated platelets, PtdOH formation was maintained even though no TXB_2 generation was observed. This implies that the formation of PtdOH was independent of thromboxane, and that collagen itself may activate this PtdOH formation, via phospholipase C activation, for instance. This is totally in agreement with previous results showing that PtdOH formation is independent of thromboxane-receptor occupancy [10,41], and that $InsP_3$ accumulates in indomethacin-treated platelets [18]. It is also noteworthy that half-maximum P43 phosphorylation and secretion were obtained in the absence of TXB_2 generation, indicating that TXB_2 formation is not absolutely required to initiate the activity of protein kinase C and the secretory processes. Furthermore, the blockade of thromboxane receptors was observed to be accompanied by a decreased, but still marked, generation of TXB_2 [42]. In agreement with other studies [41,42], our results support the possibility that collagen–platelet interaction itself contributes to the initial formation of TXB_2, which in turn might amplify platelet responses. This positive feedback of TXB_2 is completely consistent with the results of our kinetic studies.

In conclusion, the present work strongly suggests that stimulation of platelets by collagen involves the protein kinase C pathway more particularly associated with secretion. It also suggests that collagen-induced stimulation is not entirely mediated by the thromboxane pathway, at least during its initial stage.

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