Evidence for a role for tyrosine phosphorylation of phospholipase Cγ2 in collagen-induced platelet cytosolic calcium mobilization

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(1) The non-specific protein kinase C inhibitor, staurosporine, inhibited collagen-induced increases in cytosolic free Ca^{2+} while having no effect on Ca^{2+} mobilization by other platelet agonists. A more specific inhibitor of protein kinase C, Ro 31-8220, did not inhibit collagen-induced Ca^{2+} mobilization. Neither drug had an effect on platelet adhesion to collagen. (2) Staurosporine inhibited collagen-stimulated tyrosine phosphorylation, while Ro 31-8220 had no effect. (3) It also inhibited collagen-induced phosphatidic acid formation, inositol trisphosphate formation and arachidonic acid liberation. (4) Ro 31-8220 did not inhibit collagen-stimulated arachidonic acid formation, but it enhanced collagen-stimulated phosphatidic acid and inositol trisphosphate formation. (5) Immunoprecipitation of phospholipase Cγ2 (PLCγ2) with a specific antibody demonstrated that PLCγ2 was phosphorylated on tyrosine after stimulation by collagen. (6) The phosphorylation of PLCγ2 was inhibited by staurosporine but not by Ro 31-8220. These results provide additional evidence that the mechanism of signal transduction for collagen is different from other platelet agonists and indicate that it involves activation of PLCγ through a tyrosine kinase-dependent mechanism.

INTRODUCTION

After damage to the vessel wall, circulating platelets adhere to newly exposed collagen. In addition to simple attachment, this event triggers an intracellular signalling cascade similar to that invoked by other agonists that culminates in the production of thromboxane A_2 and secretion of the constituents of storage granules. These products act as secondary agonists to recruit many more platelets to consolidate the haemostatic plug. There are several candidates for a receptor mediating platelet adhesion to collagen including the integrin glycoproteins Ia/IIa, as well as glycoproteins IV and VI (Santoro et al., 1988; Tandon et al., 1989; Moroi et al., 1989).

The receptor responsible for induction of intracellular signal transduction by collagen is unknown. Two different general mechanisms for signal transduction have been described. In the first mechanism, a receptor, upon binding its ligand, interacts with a guanine-nucleotide-binding protein which can activate intracellular proteins, leading to a change in second messengers. Evidence indicates that many platelet-agonist receptors are coupled via guanine-nucleotide-binding proteins (Shenker et al., 1991; Manning and Brass, 1991). In the other mechanism, when the ligand binds to its receptor a tyrosine kinase is activated. This kinase may be a domain of the receptor itself or it may be a separate protein that is non-covalently associated with the receptor.

Compared with other tissues, platelets possess high levels of $pp60^{c\text{-}src}$, a member of a family of tyrosine kinases (Golden et al., 1986). Platelets also contain several other members of this family including the products of the c-*yes*, c-*fyn*, c-*lyn* and c-*syk* genes (Cichowski et al., 1992; Ohta et al., 1992). The hypothesis that the products of this gene family can act as indirect response mediators coupling different signal transduction systems in cells was first confirmed with T lymphocytes. In these cells it was found that the *lck* gene product, p56 lck, is physically associated with the T lymphocyte CD4 and CD8 surface receptor proteins and that clustering of these receptors causes tyrosine kinase activation (Veillette et al., 1988). Similar results were soon reported for fyn kinase and the T-cell receptor (Samuelson et al., 1990) and lyn kinase and the B-cell antigen receptor (Yamanashi et al., 1991).

While several authors have described the occurrence of tyrosine phosphorylation in platelets (Ferrell and Martin, 1988, 1989; Golden and Brugge, 1989; Golden et al., 1990), a role for this response has not yet been established. We recently developed an assay to study platelet adhesion to collagen (Smith and Dangelmaier, 1990). We showed that adhesion is associated with an increase in cytosolic Ca^{2+} and phosphatidic acid (PA) formation (Smith and Dangelmaier, 1990; Smith et al., 1991). We also found that the increase in cytosolic Ca^{2+} is necessary for collagen-induced dense-granule secretion and arachidonic acid release (Smith et al., 1992b). In contrast with other platelet agonists, collagen-induced platelet activation (Smith et al., 1992a) and tyrosine phosphorylation (Smith et al., 1993) are not inhibited by elevation of cyclic AMP. To gain insight into the mechanism of collagen-induced platelet activation, we examined the effects of the kinase inhibitors staurosporine and Ro 31-8220 on platelet adhesion to collagen and the associated collagen-induced signalling events. These include tyrosine phosphorylation, $InsP_3$ formation, PA formation and cytosolic Ca^{2+} mobilization. Our results indicate that collagen-induced platelet stimulation involves the tyrosine phosphorylation-dependent activation of phospholipase Cγ2 (PLCγ2).

EXPERIMENTAL

Materials

Fura-2 acetoxymethyl ester (Fura-2 AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.) and Sepharose 2B from Pharmacia. Human α-thrombin was a generous gift from Dr.

Abbreviations used: PLCγ2, phospholipase Cγ2; $InsP_3$, inositol trisphosphate; PA, phosphatidic acid; PAF, platelet-activating factor; mAb, monoclonal antibody; Fura-2 AM, Fura-2 acetoxymethyl ester.

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J. W. Fenton II (NY State Department of Health, Albany, NY, U.S.A.). Collagen was from Horm-Chemie (Munich, Germany) and the stable prostaglandin I_2 analogue iloprost was from Berlex Laboratories (Cedar Knolls, NJ, U.S.A.). The thromboxane A_2 receptor antagonist SQ 29548 was a gift from D. Harris of Bristol-Meyers Squibb. Arg-Gly-Asp-Ser (RGDS) was purchased from Bachem (Philadelphia, PA, U.S.A.). BSA fraction V, U46619, phosphocreatine, and creatine phosphokinase were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{14}C]5-hydroxytryptamine, [^{3}H]arachidonic acid, [^{3}H]oleic acid and [^{32}P]P_i were from Amersham (Arlington Heights, IL, U.S.A.). The monoclonal antibody (mAb) PY20, ^{125}I-protein A, and goat anti-(mouse IgG) were from ICN. Platelet-activating factor (PAF) and phenidione were obtained from Biomol (Fort Washington, PA, U.S.A.). Ro 31-8220 was a gift from Roche Products (Welwyn Garden City, Herts., U.K.). PA standard was from Avanti Biochemicals (Atlanta, GA, U.S.A.).

Platelet preparation

Human blood was taken by venipuncture from informed healthy volunteers into acid/citrate/dextrose (Verhallen et al., 1988). Platelet-rich plasma obtained by centrifugation at 180 *g* for 15 min at ambient temperature was recentrifuged at 800 *g* for 15 min. The platelet pellet was resuspended in 0.5 vol. of autologous platelet-poor plasma and incubated with 3 μM Fura-2 AM and 1 mM aspirin for 45 min at 37 °C. The platelets were separated from plasma protein and extracellular Fura-2 by gel-filtration chromatography on Sepharose 2B (Pharmacia) using a modified Ca^{2+}-free Tyrode's buffer containing 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 2 mM $MgCl_2$, 0.2 % albumin, 5 mM glucose and 10 mM Hepes (pH 7.3) (Veillette et al., 1988). Unless otherwise stated, the cell suspension was adjusted to a final concentration of 2×10^8 cells/ml.

Estimation of platelet cytosolic Ca^{2+} concentrations

Ca^{2+} measurements were made using the fluorescent indicator dye Fura-2 (Smith et al., 1991). An aliquot (1 ml) of the Fura-2-loaded platelet suspension was added to a cuvette thermostatically regulated to 37 °C and continuously stirred. Fluorescence measurements were made in a Perkin–Elmer LS-5 fluorimeter using an excitation wavelength of 340 nm and an emission wavelength of 510 nm. In order to convert fluorescence measurements into Ca^{2+} concentrations, $F_{min.}$ was determined following the addition of digitonin (50 μM) in the presence of EGTA and Tris base. $F_{max.}$ was measured by the addition of excess $CaCl_2$. Free Ca^{2+} concentration was calculated using these values and a K_d of 224 nM (Grynkiewwicz et al., 1985) after correction for extracellular dye.

Measurement of PA and InsP_3 formation

PA formation was measured in platelets that had been prelabelled with [^{32}P]P_i (Smith and Dangelmaier, 1990). Reactions were terminated with chloroform/methanol (1:2, v/v) and extracted lipids were analysed by t.l.c. on Silica Gel G plates using water-saturated ethyl acetate/iso-octane/acetic acid (9:5:2, by vol.) as solvent. A PA standard was used to identify the location of PA on the plate.

Ins(1,4,5)P_3 and Ins(1,3,4)P_3 formation were measured in platelets that had been prelabelled with [^{32}P]P_i as described above. Reactions were stopped with 0.6 M perchloric acid and processed by the method of Pulcinelli and Salganicoff (Pulcinelli et al., 1992). The radiolabelled InsP_3s were separated by h.p.l.c. as previously described (Daniel et al., 1987).

Arachidonic acid liberation

Arachidonate liberation was measured from platelets that had been prelabelled with 100 nCi/ml [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (specific radioactivity 76 Ci/mmol). Phenidione (100 μM) was added before stimulation with agonists to inhibit cyclo-oxygenase and lipoxygenase activities. Platelet suspensions were added directly to chloroform/methanol (1:2, v/v) and the extracted lipids analysed by t.l.c. on Silica Gel G plates using petroleum ether/diethyl ether/acetic acid (60:45:1, by vol.) as solvent (Smith et al., 1985).

Adhesion to collagen

For measurement of adhesion, platelets were incubated for 1 h with 5 μCi/ml [9,10-^{3}H(N)]oleic acid (specific radioactivity 8.9 Ci/mmol) simultaneously during incubation with Fura-2 AM. After measurement of collagen-induced changes in intracellular Ca^{2+} (i.e. after treatment with 50 μg/ml of collagen), the platelet suspension was applied to a 10 nm mesh nylon filter and the non-adhered platelets removed by vacuum filtration and washing with the platelet gel-filtration buffer. Adhesion was determined by counting the radioactivity trapped on the nylon filter (Smith and Dangelmaier, 1990).

Platelet 5-hydroxytryptamine secretion

For measurement of 5-hydroxytryptamine secretion, platelet-rich plasma was incubated for 30 min at 37 °C with 1 μM [2-^{14}C]5-hydroxytryptamine (50 Ci/mmol) before gel filtration. Imipramine (1 μM) was added to the gel-filtered platelets to prevent re-uptake of 5-hydroxytryptamine. Secretion was stopped with formaldehyde/EDTA according to the method of Costa and Murphy (1975) with the addition that the formaldehyde/EDTA solution was kept on ice prior to platelet addition. Samples of the supernatants were added to scintillation fluid. Release of 5-hydroxytryptamine is expressed as a percentage of the total radioactivity.

Protein phosphorylation

For measurement of protein tyrosine phosphorylation, reactions were stopped with a one-tenth volume of 6.6 M perchloric acid on ice. The pellet obtained by centrifugation was washed once with water, solubilized in SDS sample buffer, boiled for 5 min in the presence of 5 mM dithiothreitol, and subjected to SDS/PAGE on a 7.5% polyacrylamide gel at 200 V for 4 h. The proteins were then electrophoretically transferred to Immobilon-P using 10 mM Caps and 10% (v/v) methanol for 2 h at 1 A. The Immobilon was treated overnight at 4 °C with 3% (w/v) BSA (in 138 mM NaCl, 25 mM Tris/HCl, pH 8.0, containing 25 mM NaN_3 and 0.05% Tween-20) to block non-specific binding and then probed at room temperature for 2 h with a phosphotyrosine-specific mAb [PY20, 1 μg/ml (Yamanashi et al., 1991)] in 138 mM NaCl, 3% BSA, 25 mM NaN_3, 0.05% Tween-20 and 25 mM Tris/HCl (pH 8.0). The Immobilon was washed five times in the same buffer excluding the antibody. Bound mAb was detected by incubation for 1 h with ^{125}I-labelled goat anti-(mouse IgG) [2 μCi/ml in Tris-buffered saline containing 3% (w/v) albumin, 0.05% Tween-20 and 25 mM NaN_3].

This was washed ten times as described above and submitted to autoradiography.

Total platelet phosphorylation was determined by the incorporation of $[^{32}P]P_i$ into the 20 kDa myosin-light-chain band and the 47 kDa pleckstrin band on SDS/PAGE (Daniel et al., 1980).

Immunoprecipitation of PLCγ2

Gel-filtered platelets that had been pretreated with 1 mM aspirin were incubated with collagen (100 μg/ml) for 2 min in the presence of iloprost and feedback inhibitors (see legend to Figure 1 for details). Samples were filtered as in the adhesion assay. The filters (10 filters per assay sample) were placed in 1 ml of cold RIPA [50 mM NaCl, 50 mM Tris (pH 8.0), 1 mM EDTA, 1 mM EGTA, 3 mM sodium orthovanadate, 1 mM ammonium molybdate, 1 % Nonidet NP-40, 0.5 % deoxycholate, 0.1 % SDS, 1 mM phenylmethanesulphonyl fluoride and 5 μg/ml leupeptin] and allowed to sit on ice for 30 min. Anti-PLCγ2 (10 μl; a gift from Dr. Graham Carpenter, Vanderbilt University, Nashville, TN, U.S.A.) was added to the lysate and the solution incubated on ice for 1 h. Pansorbin (100 μl; pre-washed with RIPA buffer) was added and the samples rocked overnight at 4 °C. Immunoprecipitates were retrieved by brief centrifugation in a microfuge, washed once with RIPA buffer, twice with 150 mM NaCl, 10 mM NaH_2PO_4 (pH 7.6), 3 mM sodium orthovanadate and twice with 100 mM Tris (pH 7.4), 500 mM LiCl, 3 mM sodium orthovanadate. Proteins were subjected to SDS/PAGE (7.5 % polyacrylamide), transferred to Immobilon-P and probed as described in the tyrosine phosphorylation procedure. After radioautography, we stripped off the primary and detection antibodies and reprobed for the presence of PLCγ2 according to Tate and Rittenhouse (1993) except that we used ^{125}I-Protein A for detection.

RESULTS

Figure 1 shows the effect of staurosporine on agonist-dependent elevation of cytosolic Ca^{2+} in human platelets. In the case of four of the agonists: thrombin, PAF, ADP and the thromboxane analogue, U46619, staurosporine treatment had only minor effects on the Ca^{2+} transient. There was little change in the maximal Ca^{2+} concentration attained, but in the case of thrombin and PAF there was a prolongation of the transient. By contrast, staurosporine (1 μM) completely inhibited the increase in Ca^{2+} induced by collagen.

In order to differentiate whether the inhibition of collagen-induced Ca^{2+} mobilization by staurosporine was due to inhibition of protein kinase C or of tyrosine kinases, we tested the effect of the staurosporine analogue Ro 31-8220 (10 μM), which has greater specificity for protein kinase C (Davis et al., 1989; Walker and Watson, 1993). Figure 2 shows that Ro 31-8220 did not inhibit collagen-induced Ca^{2+} mobilization. In keeping with these results, arachidonic acid liberation, a response dependent upon Ca^{2+} mobilization (Smith et al., 1992b), was similarly inhibited by staurosporine but only slightly affected by Ro 31-8220 (Figure 3).

Neither Ro 31-8220 (10 μM) nor staurosporine (1 μM) inhibited adhesion of platelets to 50 μg/ml collagen (22.2 % ± 3.5 for control; 24.8 % ± 4.6 for staurosporine- and 21.7 % ± 2.2 for Ro 31-8220-treated cells). In order to be certain that Ro 31-8220 was inhibiting protein kinase C in our experiments, we compared the effects of Ro 31-8220 and staurosporine on collagen-induced

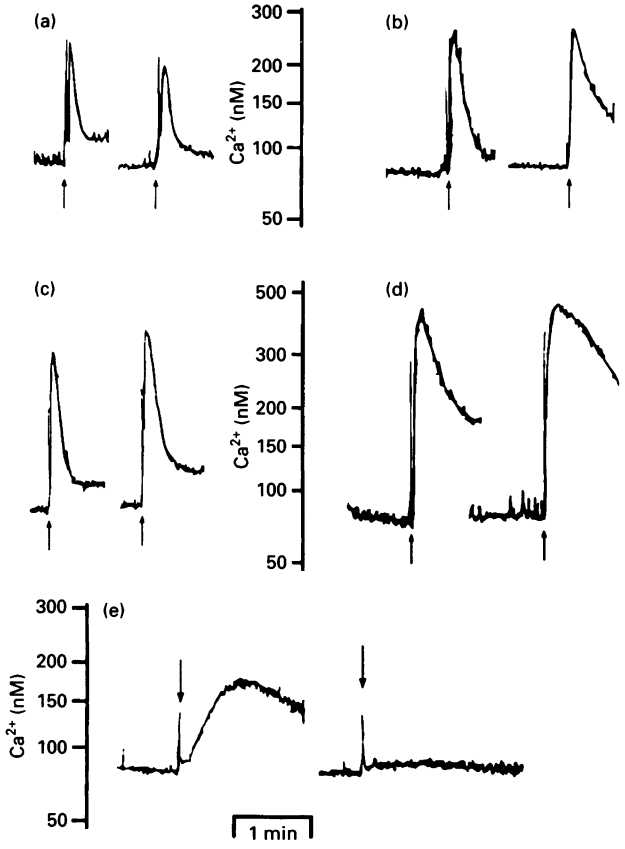

Figure 1 The effect of staurosporine on agonist-stimulated Ca^{2+} mobilization in gel-filtered human platelets

The traces indicate agonist-induced changes in fluorescence in Fura-2-labelled platelets. In each panel the left-hand trace is the control and the right-hand trace has been treated with 1 μM staurosporine for 2 min before addition of agonist. Addition of agonist is indicated by the arrows. In panel (**a**) the cells were pretreated with 5 μM SQ 29548 and 120 μM RGDS and stimulated with 10 μM ADP; in (**b**) the cells were pretreated with 50 units/ml of creatine phosphokinase, 20 mM creatine phosphate, 5 μM SQ 29548 and 120 μM RGDS and stimulated with 200 nM PAF; in (**c**) the cells were pretreated with 50 units/ml of creatine phosphokinase, 20 mM creatine phosphate and 120 μM RGDS and stimulated with 1 μM U46619; in (**d**) the cells were pretreated with 50 units/ml of creatine phosphokinase, 20 mM creatine phosphate, 5 μM SQ 29548 and 120 μM RGDS and stimulated with 1 unit/ml of thrombin; in (**e**) the samples were pretreated with 50 units/ml of creatine phosphokinase, 20 mM creatine phosphate, 5 μM SQ 29548, 120 μM RGDS and 28 nM iloprost and stimulated with 50 μg/ml collagen.

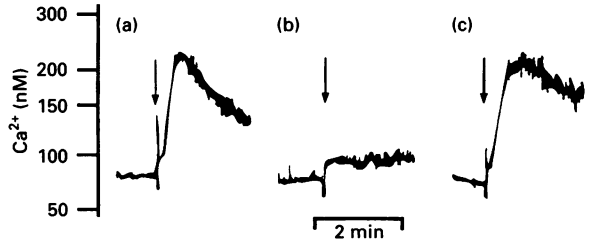

Figure 2 Comparison of the effect of Ro 31-8220 and staurosporine on collagen-induced Ca^{2+} mobilization

The traces indicate collagen-induced (50 μg/ml) changes in fluorescence in Fura-2-labelled platelets. (**a**) Control platelets; (**b**) cells treated with 1 μM staurosporine for 2 min prior to addition of collagen; (**c**) cells treated with 10 μM Ro 31-8220 for 2 min prior to addition of collagen. All three samples contained creatine phosphate, creatine phosphokinase, SQ 29548 and RGDS and 28 nM iloprost.

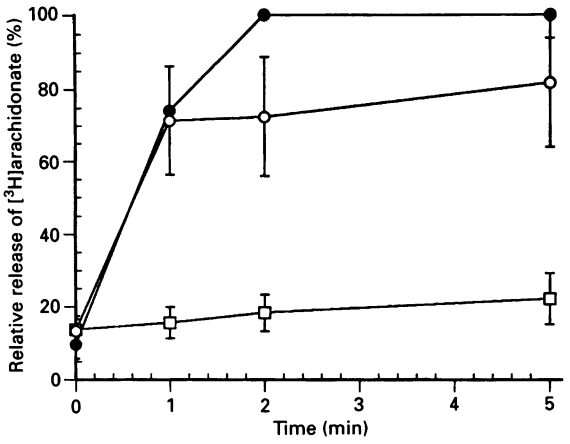

Figure 3 Comparison of the effect of Ro 31-8220 and staurosporine on collagen-induced arachidonic acid liberation

Aliquots of gel-filtered platelets were treated with collagen as shown in Figure 2 and analysed for arachidonic acid liberation as described. Platelets were either treated with vehicle (●), 1 μM staurosporine (□) or 10 μM Ro 31-8220 (○). The graph displays the mean ± S.E.M. for determinations made in three separate experiments. The values plotted were determined by setting the control value of arachidonic acid liberation at 2 min in each experiment to 100%. The relative percentage arachidonate liberation was then calculated for each sample before the data from different experiments were averaged. This calculation was necessary as the total level of incorporation of [^{3}H]arachidonic acid was different in each experiment.

secretion of dense-granule constituents and protein phosphorylation. Both compounds had the expected effect of totally inhibiting 5-hydroxytryptamine secretion and pleckstrin phosphorylation at the concentrations that were used (results not shown). In agreement with the findings of Walker and Watson (1993), myosin-light-chain phosphorylation was completely abolished by staurosporine (1 μM) while Ro 31-8220 (10 μM) had a lesser effect (results not shown).

The effects of staurosporine and Ro 31-8220 on collagen- and thrombin-induced tyrosine phosphorylation are shown in Figure 4. As we described previously (Smith et al., 1993), both thrombin and collagen enhanced tyrosine phosphorylation of platelet proteins, with collagen causing the phosphorylation of an additional 40 kDa protein. Staurosporine effectively inhibited the increases in tyrosine phosphorylation produced by both collagen and thrombin. Ro 31-8220 did not inhibit tyrosine phosphorylation induced by collagen, although it partially inhibited that induced by thrombin.

The effect of staurosporine and Ro 31-8220 on phosphatidylinositol metabolism was determined by the measurement of collagen-stimulated PA formation (Figure 5). Staurosporine completely abolished collagen-induced increases in [^{32}P]PA formation. On the other hand, Ro 31-8220 significantly enhanced the collagen-induced formation of [^{32}P]PA. A similar pattern of effects of the two compounds on collagen-induced InsP_3 formation was observed (Table 1).

These data are consistent with the hypothesis that cytosolic Ca^{2+} mobilization induced by collagen is mediated via a phospholipase C-dependent pathway acting on Ins(4,5)P_2. Recently, Tate and Rittenhouse (1993) showed that thrombin can phosphorylate PLCγ2, a form of PLC that is activated by tyrosine phosphorylation. Figure 6 shows the results obtained when platelets were lysed, immunoprecipitated with an antibody against PLCγ2, subjected to SDS/PAGE and probed for phosphotyrosine. No detectable phosphorylated PLCγ2 was found in unstimulated platelets. However, phosphorylation of PLCγ2 was high in

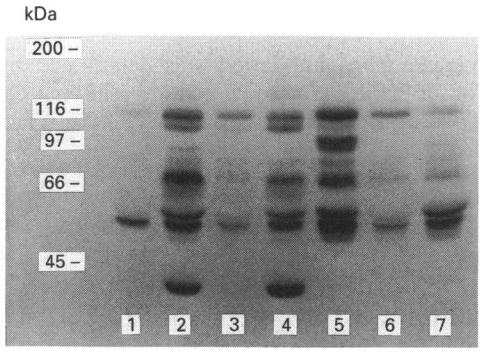

Figure 4 The effect of Ro 31-8220 and staurosporine on collagen- and thrombin-induced tyrosine phosphorylation

Aliquots of a gel-filtered platelets suspension were analysed for tyrosine phosphorylation as described in the Experimental section. Treatments are: lane 1, control; lane 2, collagen; lane 3, collagen plus staurosporine; lane 4, collagen plus Ro 31-8220; lane 5, thrombin; lane 6, thrombin plus staurosporine; and lane 7, thrombin plus Ro 31-8220.

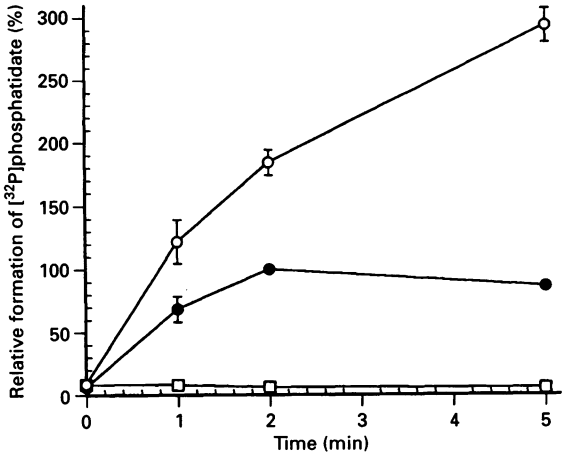

Figure 5 Comparison of the effect of Ro 31-8220 and staurosporine on collagen-induced PA liberation

Aliquots of a gel-filtered platelet suspension were treated as in Figure 3, and analysed for PA liberation as described. Symbols are the same as in Figure 3. The graph displays the mean ± S.E.M. for determinations made in two separate experiments and plotted as described in the legend in Figure 3.

Table 1 The effect of staurosporine and Ro 31-8220 on collagen-stimulated InsP_3 formation

Cells were incubated with either vehicle, 1 μM staurosporine or 10 μM Ro 31-8220 for 2 min before stimulation with 100 μg/ml of collagen for 2 min at 37 °C in the presence of 50 units/ml of creatine phosphokinase, 20 mM creatine phosphate, 5 μM SQ 29,548, 120 μM RGDS and 28 nM iloprost. The InsP_3 levels were determined as described in the Experimental section and the results are expressed as c.p.m. ± S.D. of triplicate values. The table is representative of three experiments.

	Ins(1,3,4)P_3 (c.p.m.)	Ins(1,4,5)P_3 (c.p.m.)
Control	46 ± 16	538 ± 97
Collagen	4805 ± 1022	1941 ± 469
Collagen + staurosporine	245 ± 65	686 ± 107
Collagen + Ro 31-8220	14345 ± 238	3838 ± 243

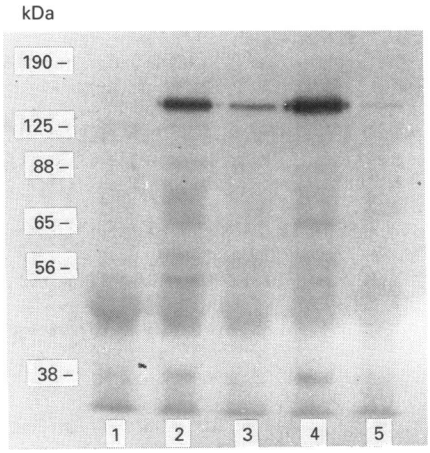

Figure 6 Collagen-induced tyrosine phosphorylation of PLCγ2 is inhibited by staurosporine but not Ro 31-8220

Platelets (10 samples of 4×10^8 cells) were pretreated either with vehicle (lane 2); 1 μM staurosporine (lane 3); or 10 μM Ro 31-8220 (lane 4). These samples were incubated with collagen (100 μg/ml) for 2 min at 37 °C and the adherent cells were separated on ten 10 μm filters, lysed and immunoprecipitated as described in the Experimental section. For control cells, i.e. not stimulated (lane 1), and cells stimulated by 5 units/ml of α-thrombin (0.5 min at 37 °C, lane 5) the extent of adhesion to collagen was determined in aliquots of the samples from lanes 2–4 and a comparable number of cells were concentrated by centrifugation, then lysed and used for immunoprecipitation. Immunoprecipitates were separated by SDS/PAGE, transferred to Immobilon and probed with PY20 antibody for the presence of phosphotyrosine. This figure is representative of three experiments.

collagen and collagen + Ro 31-8820-stimulated cells but considerably less in collagen + staurosporine-treated cells. This latter result was not due to the lack of adhesion of the staurosporine-treated cells to collagen as the level of adhesion in the presence of staurosporine was the same as the collagen-stimulated cells in its absence (see above). When this immunoblot was reprobed for the presence of PLCγ2 approximately equal amounts of PLCγ2 were found in all samples. For comparison, Figure 6 (lane 5) shows the relative phosphorylation of PLCγ2 induced by thrombin (5 units/ml). It is apparent that the level of PLCγ2 phosphorylation is substantially less in the thrombin-stimulated cells compared with collagen-stimulated cells. Note that about 5-fold more platelets were required by Tate and Rittenhouse (1993) to demonstrate thrombin-induced PLCγ2 phosphorylation than we have used here.

DISCUSSION

Collagen appears to be similar to other platelet agonists in that it can directly mobilize cytosolic Ca^{2+}, activate phospholipase C (as shown by increased PA and $InsP_3$ formation), liberate arachidonic acid and cause secretion of dense granule constituents. However, collagen is different from other agonists in two important respects. First collagen is unique when compared with thrombin, ADP or the thromboxane A_2 mimetic U46619 in that collagen-induced platelet activation is not inhibited by elevation of cytosolic cyclic AMP (Smith et al., 1992a, 1993). Secondly, as shown here, collagen-induced Ca^{2+} mobilization is inhibited by staurosporine while that induced by several other platelet agonists is not. These results suggest that there is a fundamental difference between the initial steps of the collagen-mediated signal transduction pathway and those of other agonists.

Recent studies from several laboratories have focused on the role of protein-tyrosine phosphorylation in platelet activation. These studies have been spurred by the finding of high levels of tyrosine kinases in platelets including $pp60^{c\text{-}src}$, $pp60^{fyn}$, $pp54,58^{lyn}$, $ppy60^{hck}$, $pp60^{yes}$ and $p72^{syk}$ (Golden et al., 1986; Horak et al., 1990; Huang et al., 1991a; Ohta et al., 1992). It has been shown by Ferrell and Martin (1988) and Golden and Brugge (1989) that thrombin treatment induces rapid changes in tyrosine phosphorylation of a number of platelet proteins. Phosphorylation of a subset of these proteins, with reported molecular masses of 126, 108 and 100 kDa (Ferrell and Martin, 1989) or 97, 95, and 84 kDa (Golden et al., 1990) depends on the presence and activation of glycoprotein IIb/IIIa. This conclusion is based on the fact that this subset of proteins is not phosphorylated in a patient with Glanzmann thrombasthenia lacking GPIIb/IIIa. This phosphorylation event is also inhibited by peptides such as RGDS and mAbs that inhibit the binding of fibrinogen to GPIIb/IIIa.

The role of tyrosine phosphorylation in platelet secretion is unclear. Golden et al. (1990) found that tyrosine phosphorylation of the 84, 95, and 97 kDa proteins was not required for secretion by several agonists including collagen. However, as has been the case for most previous studies with collagen, the authors failed to inhibit positive feedback and used low concentrations of collagen. Thus in their study tyrosine phosphorylation and secretion were most likely produced by ADP and thromboxane A_2 rather than by collagen itself. Rendu et al. (1992) found that tyrosine kinase inhibitors such as tryphostin AG 213 were capable of inhibiting thrombin-induced secretion at relatively low thrombin concentrations. Despite repeated attempts, we were unable to demonstrate the effect of any of a variety of tyrosine kinase inhibitors, including AG 213 and genistein, on either thrombin- or collagen-induced Ca^{2+} mobilization and tyrosine phosphorylation. In the current study, we found inhibition of tyrosine phosphorylation with staurosporine had no major effect on Ca^{2+} mobilization with thrombin, ADF, PAF or a thromboxane analogue, suggesting that tyrosine phosphorylation does not play a primary role in signal transduction for these agonists, at least at relatively high concentrations of agonist.

Kornberg et al. (1991) have shown that clustering of integrins with an antibody to the β1 integrin subunit leads to the tyrosine phosphorylation of a 130 kDa protein. They have proposed that signal transduction via integrins may occur primarily by tyrosine phosphorylation. Pumiglia et al. (1992) and Blake et al. (1993) found that artificial stimulation of tyrosine phosphorylation with tyrosine phosphatase inhibitors was capable of activating platelets, indicating that the tyrosine phosphorylation does have the potential of being a primary pathway in platelet signal transduction. We attribute the inhibition noted here of collagen-induced Ca^{2+} mobilization by staurosporine to its ability to inhibit tyrosine kinases rather than to an effect on protein kinase C. Protein kinase C activation is integral to platelet secretion by all agonists, whereas the effect of staurosporine is restricted to collagen. More importantly, the specific protein kinase C inhibitor Ro 31-8220 had no effect on collagen-induced Ca^{2+} mobilization at concentrations where it inhibited phosphorylation of pleckstrin and completely abolished 5-hydroxytryptamine secretion. In agreement, Watson et al. (1993) have reported similar results in abstract form. The inhibition of thymocyte proliferation by staurosporine has been previously attributed to an action on both protein kinase C and tyrosine kinase (Zilberman and Gutman, 1992). Of course, the evidence gained using kinase inhibitors must be viewed with some caution as these drugs may have additional effects.

Finally, we suggest that collagen may have a primary action to

activate PLCγ. Consistent with this idea, we have shown that staurosporine inhibits collagen-induced formation of InsP_3s and PA. We show here that collagen also causes tyrosine phosphorylation of PLCγ2 and that this phosphorylation is inhibited by staurosporine but not Ro 31-8220. Previous authors have shown that staurosporine does not inhibit PA formation induced by thrombin (Huang et al., 1991b). This finding suggests that the primary pathway for thrombin is through another PLC isoenzyme and is consistent with our observation that thrombin induces a weaker phosphorylation of PLCγ2 than collagen. The effect of the protein kinase C inhibitor Ro 31-8220 in enhancing InsP_3 and PA formation is consistent with the finding that activation of protein kinase C inhibits PA formation (MacIntyre et al., 1985). In addition, another inhibitor of protein kinase C, Ro 31-7549, has been shown to potentiate activation of PLCγ in rat RBL-2H3 cells (Ozawa et al., 1993). The results presented here indicate that collagen-dependent platelet responses occur as a result of the tyrosine phosphorylation-dependent activation of PLCγ2. Proof of this hypothesis awaits the elucidation of the exact processes involved in the collagen-activation pathway.

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