Cytosolic calcium as a second messenger for collagen-induced platelet responses

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We showed previously that direct platelet activation by collagen involves an increase in the platelet cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) but that this increase is not required for the adhesion of platelets to collagen. We now report that collagen-induced arachidonic acid liberation, myosin phosphorylation and 5-hydroxytryptamine secretion are dependent on increases in $[\text{Ca}^{2+}]_i$, as they were markedly inhibited in platelets loaded with the acetoxymethyl ester of the Ca^{2+} chelator BAPTA but not in cells loaded with the acetoxymethyl ester of the non-chelating diazo-3. BAPTA also partially inhibited the rate of collagen-induced phosphatidic acid (PtdA) formation but had little effect on increases in phosphorylation of pleckstrin (47 kDa protein; P47). From these results we infer that collagen-induced increases in $[\text{Ca}^{2+}]_i$ are required for dense granule secretion and arachidonic acid liberation, but are not necessary for stimulation of the protein kinase C pathway.

INTRODUCTION

Considerable evidence had accumulated that increases in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) are involved in all of the major responses of platelets to stimulation, including shape change, aggregation, secretion and release of arachidonic acid from phospholipids (see [1,2] for recent reviews). A rapid receptor-mediated elevation in $[\text{Ca}^{2+}]_i$ has been demonstrated for many platelet agonists, including thrombin, ADP, thromboxane A_2 , platelet-activating factor and vasopressin. By contrast, it was suggested that collagen causes arachidonic acid liberation by mechanisms which do not involve an elevation of $[\text{Ca}^{2+}]_i$ [3].

Several platelet membrane proteins have been proposed as the platelet receptor for collagen, but the weight of evidence favours the heterodimeric complex of glycoprotein Ia/IIa (GPIa/GPIIa) ($\alpha_2\beta_1$). Patients whose platelets are deficient in GPIa have a mild bleeding disorder, and their platelets show impaired aggregation in response to collagen but not to other agonists [4]. Moreover, the GPIa/GPIIa complex can be purified by affinity chromatography on a collagen–Sepharose column [5]. After incorporation of this solubilized complex into liposomes, the liposomes adhere to collagen [6]. Previously we reported the development of an assay to measure the adhesion of platelets to collagen in the presence of a receptor antagonist to prostaglandin endoperoxides and thromboxane A_2 as well as an ADP-removing system [7]. Cytosolic Ca^{2+} mobilization induced directly by high concentrations of collagen was detected in this assay using fura-2 as an intracellular indicator [7], and subsequently was found to depend on the rapid energy- and actin polymerization-dependent adhesion of the platelets to the collagen [8]. This increase in $[\text{Ca}^{2+}]_i$ was unique when compared with that induced by other agonists in that it was not inhibited by elevation of cyclic AMP [9]. In the present paper we report on the importance of cytosolic Ca^{2+} as a second messenger in collagen-induced stimulus–response coupling.

EXPERIMENTAL

Human blood was obtained from healthy volunteers who denied taking any drug during the previous week. The blood was anticoagulated with citric acid/citrate/dextrose [10] and centri-

fuged at 180 *g* for 15 min at room temperature to obtain platelet-rich plasma. The platelet-rich plasma was centrifuged at 800 *g* for 20 min at room temperature to obtain a platelet pellet. For most experiments, the pellet was resuspended in 0.5 vol. of autologous plasma and incubated at 37 °C with 5 μCi of [9,10- ^3H]oleic acid/ml (8.9 Ci/mmol), 5 μCi of [5,6,8,9,11,12,14,15- ^3H]arachidonic acid/ml or 0.25 mCi of [^{32}P]PO $_4$ (carrier free)/ml. After 15 min of incubation, the sample was divided into three portions: 100 μM -BAPTA/AM was added to one portion, 100 μM -diazo-3/AM to another, and a corresponding volume of the vehicle, dimethyl sulphoxide, to the third [11]. In some experiments, fura-2/AM (3 μM) also was added to all three samples. In all experiments, aspirin (1 mM) was added after 30 min of incubation. After a total incubation of 60 min at 37 °C, the platelets were separated from unincorporated radiolabel by gel filtration on three separate Sepharose 2B columns using calcium-free Tyrode's buffer supplemented with 0.2% fatty-acid-free BSA [12]. The platelets were adjusted to 2×10^8 cells/ml and Iloprost (28 nM) was added. Samples of 1 ml were stirred at 800 rev./min at 37 °C for 1 min before the addition of feedback inhibitors (220 μM -Arg-Gly-Asp-Ser, 20 mM-phosphocreatine, 50 units of creatine kinase/ml and 6 μM -SQ 29,548) and 50 μg of collagen/ml as described elsewhere [9].

Fura-2 fluorescence was monitored continuously using settings of 340 nm (excitation) and 510 nm (emission). Fura-2 signals were calibrated as described elsewhere [7]. F_{max} was determined by lysing the cells with 40 μM -digitonin in the presence of saturating CaCl_2 . F_{min} was determined by the addition of 2 mM-EGTA and 20 mM-Tris base.

Adhesion of [^3H]oleic acid-labelled platelets was determined after vacuum filtration through a 10 μm nylon mesh. Blanks were included to determine non-specific trapping of the platelets on the mesh as described elsewhere [7].

For measurement of 5-hydroxytryptamine secretion, three separate samples of autologous platelet-rich plasma containing BAPTA, diazo-3 or vehicle as described above were incubated for 30 min at 37 °C with 1 μM -[G- ^3H]5-hydroxytryptamine (8 Ci/mmol) before gel filtration. Secretion was stopped in formaldehyde/EDTA according to the method of Costa & Murphy [13] and samples of the supernatants were added to scintillation fluid. In each set of experiments the total 5-

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; GPIa, glycoprotein Ia; PtdA, phosphatidic acid; PtdCho, phosphatidylcholine.

hydroxytryptamine content of the platelets was measured by adding platelet suspension to the stopping solution, from which a sample was transferred to the counting solution. Release of 5-hydroxytryptamine is expressed as a percentage of the total 5-hydroxytryptamine content.

Arachidonic acid liberation was measured in platelets that had been prelabelled with [^3H]arachidonic acid. For measurements of arachidonic acid liberation, BW 755C was added prior to stimulation with collagen to inhibit cyclo-oxygenase and lipoxygenase activities [14]. After incubation, platelet suspensions were added directly to chloroform/methanol (1:2, v/v) and the lipids were extracted as described [14]. The extracts 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 the solvent system. A standard was used to identify the location of arachidonic acid on the plate.

Myosin phosphorylation was measured by quantification of Coomassie Blue staining of phosphorylated and dephosphorylated light chain on alkaline/urea gels [15]. Phosphatidic acid (PtdA) formation and phosphorylation of pleckstrin [16] were measured in platelets that had been prelabelled with [^{32}P]P_i. For PtdA, reactions were stopped with chloroform/methanol (1:2, v/v) and the extracted lipids were analysed by t.l.c. on Silica Gel G plates using chloroform/methanol/acetic acid/water (81:10:4.5:5) as solvent. A PtdA standard was used to identify the location of PtdA on the plate. Phosphorylation of pleckstrin was measured as the incorporation of [^{32}P]P_i into the 47 kDa band on SDS/PAGE [17].

Radiolabelled [5,6,8,9,11,12,14,15- ^3H (n)]arachidonic acid (76 Ci/mmol) was from New England Nuclear, 5-hydroxy[side chain-2- ^{14}C]tryptamine creatinine sulphate (52 Ci/mol) and [9,10- ^3H (n)]oleic acid (10 Ci/mmol) were from Amersham and carrier-free [^{32}P]P_i was from ICN. Collagen was from Horm-Chemie, Munich, Germany, and Arg-Gly-Asp-Ser (RGDS) was

from Calbiochem Corporation, La Jolla, CA, U.S.A. The antagonist SQ 29,548 was a gift from D. Harris of Bristol-Myers Squibb. The stable prostaglandin I₂ analogue Iloprost was from Berlex Laboratories Inc., Cedar Knolls, NJ, U.S.A. BAPTA, diazo-3 and fura-2 acetoxymethyl esters were from Molecular Probes, Junction City, OR, U.S.A., and the nylon mesh was from Small Parts Inc., Miami, FL, U.S.A.

RESULTS

Fig. 1 shows that collagen-induced increases in $[\text{Ca}^{2+}]_i$ were eliminated in BAPTA-loaded platelets as compared with control platelets or platelets loaded with diazo-3. In contrast, neither BAPTA nor diazo-3 significantly influenced the extent of adhesion of platelets to collagen, as indicated by the percentage

Fig. 2. Secretion of 5-hydroxytryptamine induced by collagen (50 µg/ml) in suspension

The platelets were untreated (●), treated with 100 µM-BAPTA/AM (□) or treated with 100 µM-diazo-3/AM (○). The experimental conditions were identical to those used in Fig. 1. The results are representative of four experiments. Values are corrected for extracellular 5-hydroxytryptamine present before the addition of agonist.

Fig. 1. Collagen-induced increases in $[\text{Ca}^{2+}]_i$

Ca^{2+} mobilization, as measured with fura-2, was induced by a collagen suspension (50 µg/ml) in the presence of phosphocreatine/creatine kinase, SQ 29,548 and RGDS in control cells (a), or in cells preincubated for 45 min at 37 °C with 100 µM-BAPTA/AM (b) or 100 µM-diazo-3/AM (c). The tracings are representative of three experiments. The percentage adhesion of platelets to the collagen was measured at 5 min and is indicated by the numbers in the ovals above each trace.

Fig. 3. Arachidonic acid liberation induced by collagen (50 µg/ml) in suspension

The platelets were untreated (●), treated with 100 µM-BAPTA/AM (□), or treated with 100 µM-diazo-3/AM (○). The experimental conditions were identical to those used in the other Figures. In this experiment, representative of three experiments, there were 60 790 c.p.m. present in the phospholipids of the control cells at zero time.

Fig. 4. Myosin phosphorylation induced by collagen (50 µg/ml) in suspension

The platelets were untreated (●), treated with 100 µM-BAPTA/AM (□) or treated with 100 µM-diazo-3/AM (○). The experimental conditions were identical to those used in the other Figures. The experiment shown is representative of three separate experiments.

Fig. 5. PtdA formation induced by collagen (50 µg/ml) in suspension

The platelets were untreated (●), treated with 100 µM-BAPTA/AM (□) or treated with 100 µM-diazo-3/AM (○). The experimental conditions were identical to those used in the other Figures. Shown are the means $\pm$ S.D. of three separate experiments. In each experiment the largest increase in PtdA obtained regardless of the pretreatment (which ranged from 3786 c.p.m. to 7053 c.p.m.) was taken to be 100%.

adhesion shown in the ovals above each tracing. In these experiments, performed with 50 µg of collagen/ml in the presence of the adenylate cyclase stimulator Iloprost, the majority of this adhesion occurred in the first 1 min of incubation.

Fig. 2 shows that, under identical experimental conditions as above, control platelets secreted 5-hydroxytryptamine in response to collagen. As approx. 50% of the platelets adhered to collagen under these conditions (Fig. 1), it can be calculated that over 50% of the 5-hydroxytryptamine content of the adhered platelets had been secreted by 10 min at 37 °C. This secretion was only slightly inhibited in diazo-3-loaded platelets, but was inhibited by 85% in BAPTA-loaded cells. Thus collagen-induced secretion of dense granule constituents is linked to increases in $[Ca^{2+}]_i$ (Fig. 1). Fig. 3 shows that, as with 5-hydroxytryptamine secretion, liberation of arachidonic acid from phospholipids was dependent on increases in $[Ca^{2+}]_i$. Approx. 10% of the radio-

Fig. 6. Pleckstrin (P47) phosphorylation induced by collagen (50 µg/ml) in suspension

The platelets were untreated (●), treated with 100 µM-BAPTA/AM (□) or treated with 100 µM-diazo-3/AM (○). The experimental conditions were identical to those used in the other Figures. Shown are the means $\pm$ S.D. of three separate experiments. In each experiment the largest increase in pleckstrin obtained regardless of the pretreatment (which ranged from 873 c.p.m. to 1237 c.p.m.) was taken to be 100%.

labelled arachidonic acid present in the phospholipids of the adherent platelets was liberated during 10 min of incubation with collagen in the control cells or the diazo-3-loaded cells. This was reduced to 0.5% in BAPTA-loaded platelets, a 95% reduction.

The phosphorylation of myosin light chain induced by collagen peaked only slightly later than the peak in $[Ca^{2+}]_i$ (Fig. 1) in both the control and diazo-3-preloaded cells (Fig. 4). Only a small increase in phosphorylation of myosin light chain was detected in BAPTA-preloaded platelets.

While the initial rate of PtdA formation was identical in controls and diazo-3-treated platelets, it was decreased by about 40% in the BAPTA-preloaded cells (Fig. 5). BAPTA had no effect on the phosphorylation of pleckstrin (Fig. 6). Diazo-3 also did not affect the initial rate of pleckstrin phosphorylation.

DISCUSSION

In the experiments presented here, the function of the collagen-induced increases in $[Ca^{2+}]_i$ was probed by preloading the platelets with the membrane-permeant ester of the EGTA analogue BAPTA [11]. Once inside the cell, this derivative is cleaved by non-specific cytosolic esterases, leaving the membrane-impermeant tetra-anion trapped in the cytosol. When cells are loaded with BAPTA at levels that perturb normal cellular Ca^{2+} movements by chelation, it is imperative that possible toxic effects of by-products (acid and formaldehyde) of the cleavage reaction be critically assessed. To control for this, we also preincubated platelets with diazo-3/AM, an acetoxymethyl ester that does not chelate Ca^{2+} once it has been cleaved [11]. In addition, since the excitation wavelength of BAPTA is sufficiently different from that of fura-2, fluorescence changes in the latter could be used to establish that BAPTA eliminated increases in $[Ca^{2+}]_i$ (Fig. 1). Davies and co-workers [18] previously showed that by preloading platelets with dimethyl-BAPTA they could abolish thrombin-induced increases in $[Ca^{2+}]_i$, as measured with indo-1, and that these platelets no longer exhibited cytosolic alkalinization, membrane depolarization or secretion of the lysosomal granule constituent β -glucuronidase.

Previous workers [3,19,20] have been unable to detect increases in $[Ca^{2+}]_i$ in platelets in response to collagen when the cells were

pretreated with indomethacin. In all of these studies, collagen was used at a maximum concentration of 20 $\mu\text{g}/\text{ml}$. We subsequently reported, however [9], that both platelet adhesion and increases in $[\text{Ca}^{2+}]_i$ rose in proportion to the collagen concentration up to 100 $\mu\text{g}/\text{ml}$, and that, at 20 μg of collagen/ml, less than 20 % of the platelets adhered and $[\text{Ca}^{2+}]_i$ increased less than 2-fold. Moreover, in earlier studies [19,20], collagen-induced changes in $[\text{Ca}^{2+}]_i$ were measured in quin2-loaded cells. Since quin2 has a 2-fold lower K_d for Ca^{2+} than fura-2, and since it was used at substantially higher concentrations than fura-2, it is likely that quin2 buffered cytosolic Ca^{2+} in a manner similar to BAPTA.

The results in Fig. 1 are consistent with our previous observations that BAPTA has little effect on platelet adhesion [8], and show that this is also true for diazo-3. In a separate series of experiments we observed that the adhesion of electrically permeabilized platelets to collagen was relatively constant over a wide range of Ca^{2+} concentrations, indicating that adhesion does not depend on changes in $[\text{Ca}^{2+}]_i$ [21]. Adhesion of such platelets to collagen was markedly enhanced by addition of stable GTP analogues and was inhibited by the stable GDP analogue guanosine 5'-[β -thio]diphosphate, suggesting a role for guanine nucleotide regulatory proteins in the adhesive process.

BAPTA markedly inhibited both 5-hydroxytryptamine secretion and arachidonic acid liberation induced by collagen, as shown in Figs. 2 and 3. A possible explanation for the inhibition of dense granule secretion by chelation of intracellular Ca^{2+} is that activation of myosin light chain kinase by Ca^{2+} is required for myosin light chain phosphorylation [15] and activation of a contractile process that produces exocytosis. As shown in Fig. 4, we observed that, even in the presence of the feedback inhibitors, an increased amount of phosphorylated myosin light chain was detectable within 30 s of addition of collagen in both control and diazo-3-treated cells. Little change in phosphorylated myosin light chain was detected when cells preloaded with BAPTA were challenged with collagen. Other possible mechanisms of exocytosis, and the roles played by both increases in $[\text{Ca}^{2+}]_i$ and GTP regulatory proteins, have been discussed elsewhere [22].

With regard to Fig. 3, it is well known that the phospholipase A_2 required for arachidonate liberation is a Ca^{2+} -dependent enzyme [23]. Interestingly, in earlier studies quin2 loading of platelets was used to demonstrate the importance of increases in $[\text{Ca}^{2+}]_i$ in the thrombin-induced activation of phospholipase A_2 [24]. There is also evidence that arachidonic acid liberation is dependent on GTP regulatory proteins and the protein kinase C pathway [25]. The present work suggests that activation of such proteins alone would be insufficient to activate phospholipase A_2 and that Ca^{2+} plays a very important role. On the other hand, previous studies indicate that a factor in addition to Ca^{2+} is required for the liberation of arachidonic acid in human platelets [26].

The reason for the diminished formation of PtdA in BAPTA-pretreated platelets (Fig. 5) is not clear. It is possible that this reflects inhibition, either directly by BAPTA [27] or by chelation of intracellular Ca^{2+} , of that component of PtdA formation which is attributable to phospholipase D, an enzyme that acts mainly on phosphatidylcholine (PtdCho) [28]. However, as very little PtdCho is labelled in platelets during 60 min incubation with [^{32}P], this hypothesis is unlikely. On the other hand, the level of PtdA is controlled by diacylglycerol kinase, phosphatidate phosphohydrolase and CTP:PtdA cytidyltransferase, all of which could be controlled by BAPTA *per se* or by removal of Ca^{2+} . It seems unlikely that the diminished formation of PtdA was due to activation of this latter enzyme, as the incorporation of ^{32}P into PtdIns during collagen stimulation is not influenced by BAPTA (J. B. Smith, unpublished work). The fact that the

protein kinase C-dependent phosphorylation of pleckstrin [16] was not inhibited by BAPTA (Fig. 6) suggests that BAPTA does not inhibit the phospholipase C-dependent formation of diacylglycerol, the activator of this protein kinase [17]. Of course, the possibility exists that maximal stimulation of protein kinase C is achieved at lower concentrations of diacylglycerol than obtained in these experiments, in which case the partial inhibition of PtdA formation by BAPTA could be explained by partial inhibition of phospholipase C.

The results of the present studies are consistent with the concept that activation of protein kinase C is dependent on occupation of specific receptors [29] rather than on the liberation of arachidonic acid [30]. Studies with the calcium ionophore A23187 [29] showed that its ability to activate phospholipase C and cause pleckstrin phosphorylation was prevented by both inhibiting prostaglandin endoperoxide/thromboxane A_2 synthesis with aspirin and removing ADP, although its ability to liberate arachidonic acid was not. We found that collagen-induced phosphorylation of pleckstrin was not dependent on increases in $[\text{Ca}^{2+}]_i$, while secretion of dense granule contents and secretion of arachidonic acid were. Moreover, collagen receptors, and not receptors for prostaglandin endoperoxides, thromboxane A_2 or ADP, were occupied to activate the protein kinase C pathway in the present studies. The precise role of this $[\text{Ca}^{2+}]_i$ -independent pathway in collagen-induced platelet responses remains to be elucidated.

This work was supported in part by the NIH (Grant HL-36579).

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Received 17 February 1992/12 June 1992; accepted 2 July 1992