Green tea epigallocatechin-3-gallate inhibits platelet signalling pathways triggered by both proteolytic and non-proteolytic agonists

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Summary
Epigallocatechin-3-gallate (EGCG), a component of green tea, inhibits human platelet aggregation and cytosolic \([\text{Ca}^{2+}]_c\) increases more strongly when these processes are induced by thrombin than by the non-proteolytic thrombin receptor activating peptide (TRAP), thromboxane mimetic U46619, or fluoroaluminate. In line with the previously demonstrated EGCG anti-proteolytic activity, a marked inhibition on aggregation is obtained by pre-incubation of thrombin with EGCG prior to addition to cellular suspension. The catechin also reduces cellular \(\text{Ca}^{2+}\) influx following thapsigargin-induced calcium emptying of endoplasmic reticulum, and the agonist-promoted cellular protein tyrosine phosphorylation. Both tyrosine kinases Syk and Lyn, immuno-precipitated from stimulated platelets, are greatly inhibited upon cellular pre-incubation with EGCG, which also inhibits the in vitro auto-phosphorylation and exogenous activity of these two enzymes purified from rat spleen. Both thrombin-induced aggregation and \([\text{Ca}^{2+}]_c\) increase are reduced in platelets from rats that drank green tea solutions. It is concluded that EGCG inhibits platelet activation, by hindering the thrombin proteolytic activity, and by reducing the agonist-induced \([\text{Ca}^{2+}]_c\) increase through inhibition of Syk and Lyn activities.

Keywords
Epigallocatechin-3-gallate (EGCG), thrombin proteolytic activity, platelet cytosolic \(\text{Ca}^{2+}\), non-receptor tyrosine kinases

Introduction
Platelets play a pivotal role in haemostasis and arterial thrombosis through their activation triggered by several different agonists. Most of these bind to specific membrane receptors and, through interaction with heterotrimeric G proteins, stimulate phospholipase C (PLC), and phospholipase A₂ (PLA₂), and inhibit the adenylate-cyclase-dependent cAMP formation (1-4). PLC causes the hydrolysis of phosphatidylinositol 4,5-bis-phosphate to yield 1,2-diacylglycerols, which activate protein kinase C (PKC), and inositol trisphosphate (IP₃), which induces \(\text{Ca}^{2+}\) efflux from intracellular stores. Emptying of intracellular deposits triggers a cellular \(\text{Ca}^{2+}\) influx and increase of cytosolic calcium ion concentration (\([\text{Ca}^{2+}]_c\)) (5), which activates multiple enzymes that bring about platelet secretion and aggregation. PLA₂ produces arachidonic acid, which is converted in endoperoxides and thromboxanes that reinforce platelet activation. The latter process is also accompanied by an increased activity of various protein tyrosine kinases (PTKs) and Tyr phosphorylation of a variety of proteins (6, 7).
Thrombin is one of the most potent platelet agonists and its mode of action has been closely investigated. It has been demonstrated that its proteolytic activity is essential for platelet activation, and that it triggers cellular responses through cleavage of specific receptors (PARs) and generation of a new N-terminal sequence which functions as a tethered ligand, interacting with an extra-cellular loop of the receptor and activating PLC and PLA$_2$ (1, 8-11).

Synthetic peptides reproducing the new amino terminal – thrombin receptor activating peptides (TRAPs) – have been shown to mimic thrombin activation properties (9, 12). Platelet stimulation can also be induced by direct activation of G proteins with fluoroaluminate (13), by stimulation of PKC with phorbol esters or exogenous diacylglycerols, or by artificial increase of the [Ca$^{2+}$]c obtained by treatment with Ca$^{2+}$-ionophores (14).

A large number of physiological and pharmacological compounds have been found to inhibit platelet activation (15). The list includes the green tea catechins and its major polyphenolic constituent (-)epigallocatechin-3-gallate (EGCG), which have recently been shown to prevent death due to pulmonary thrombosis in mice, and to display anti-thrombotic activity and inhibitory effect on platelet activation and cytoplasmic calcium increase induced by collagen or ADP (16, 17). EGCG and other green tea catechins have been proposed as antitumor compounds exerting anti-metastatic, -angiogenic, -inflammatory, -cardiovascular disorder effects (18-22). Preliminary studies by our group have recently shown that thrombin proteolytic activity on synthetic substrate is restrained by EGCG (22). This was not an unexpected result: in fact a number of enzymes, whether metallo- or serine-protease, have been recently reported to be sensitive – though with different IC$_{50}$ – to inhibition by EGCG (19, 20, 22).

In order to clarify the mode of anti-thrombotic action of EGCG, we investigated the effect of the phytofactor on various parameters associated to thrombin-induced platelet activation in comparison with that on activation elicited by TRAP and other agonists, including fluoroaluminate, the calcium-ionophore ionomycin, and the activator of PKC phorbol ester, which activate platelets without interacting with membrane receptors.

Materials and methods

Chemicals

Apyrase, prostacyclin, human plasma α-thrombin, thrombin receptor activating peptide SFLLRNPNDKYEPF (TRAP), phorbol myristate acetate (PMA), thapsigargin and acetylseralysyl acid were purchased from Sigma-Aldrich; fura 2/AM, ionomycin, thromboxane mimetic U46619, protease inhibitor cocktail and bovine serum albumin were from Calbiochem. [32P]Pi and [γ-32P]ATP were from Amersham Pharmacia Biotech. Anti-phosphotyrosine monoclonal antibody was from ICN Biotechnology; and anti-Lyn polyclonal antibody, raised against protein residues 44-63, from Santa Cruz Biotechnology. The peptides cdc2(6-20) (KVEKIGFG-TYGVVVK), hematopoietic lineage cell-specific protein 1 (HS1) (388-402) (EQEDPEGDYEEVLE) and angiotensin II were kindly provided by Dr. O. Marin (University of Padova, Italy). Recombinant α-synuclein was expressed and purified as described elsewhere (23). EGCG, from Calbiochem, was dissolved in distilled water, divided into small aliquots and stored at -20°C. Before use, gelatin-zymography was used to verify the inhibitory potential of the flavanol against gelatinases MMP-2 and MMP-9 (20), and against leukocyte elastase challenged with its synthetic substrate N-methoxysuccinil-Ala-Ala-Pro-Val-p-nitroanilide, as previously described (22). All other reagents were of analytical grade.

Preparation of platelet suspension

Blood samples were collected from healthy volunteers, with their informed consent and in accordance with the Helsinki declaration. Blood was immediately mixed with one-sixth volume of citric-anticoagulant (85 mM sodium citrate, 70 mM citric acid, 110 mM dextrose, pH 6.5), supplemented with 20 µg/mL apyrase and 0.8 µg/mL prostacyclin, and centrifuged for 20 min at 200 g. The supernatant platelet-rich plasma was further centrifuged for 20 min at 750 g. The spun down platelets were re-suspended in basal buffer consisting of 145 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 10 mM glucose, and 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), (pH 7.4), at a concentration of 2x10$^8$ cells/mL. Acetylsalicylic acid – freshly dissolved in 0.1 M NaHCO$_3$ – was added at 0.1 mM to inhibition by EGCG (19, 20, 22).

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Determination of the cytosolic Ca$^{2+}$ concentration

[Ca$^{2+}$]c was determined as previously reported (14). Briefly: platelets were loaded with the fluorescent probe fura 2 acetoxyethyl ester (fura 2/AM), and re-suspended at a concentration of 2x10$^8$ cells/mL in the basal buffer containing 0.5 mM Ca$^{2+}$. The fluorescence changes were measured at 36°C in a thermostatted, magnetically stirred cuvette using excitation and emission wavelengths of 340 nm and 505 nm, respectively. The loading of fura in the rat platelets was performed following the procedure previously reported (24).

Platelet aggregation

Platelet aggregation was followed turbidometrically as previously described (14). Briefly: platelets were loaded with the fluorescent probe fura 2 acetoxyethyl ester (fura 2/AM), and re-suspended at a concentration of 2x10$^8$ cells/mL in the basal buffer containing 0.5 mM Ca$^{2+}$.

Assay of platelet protein 32P-phosphorylation

32P-incorporation in platelet proteins was followed as described elsewhere (14). Briefly: platelets were loaded with [32P]-
orthophosphate (0.4 mCi/mL, 90 min at 37°C), and treated according to the experimental protocol. The incubations were terminated by the addition of one tenth volume of 3 M perchloric acid. Pelleted proteins were dissolved in 0.1 mL of buffer containing 0.6 M [tris(hydroxymethyl)aminomethane] (Tris)-HCl (pH 6.8), 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 2% (w/v) sodium dodecyl sulfate (SDS), boiled for 5 min and subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) on vertical slabs. The gels were analyzed using a Packard Instant Imager and autoradiographed on Kodak X-Omat S film.

Detection of protein tyrosine phosphorylation in cells

Platelets (15 x 10⁶) were solubilized in 65 μL of buffer containing 50 mM Tris/HCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 380 mM glycine, 2% SDS and 1% β-mercaptoethanol, pH 8.9. After 5 min treatment at 100°C, the solubilized proteins were subjected to SDS-PAGE (10% gels) and immediately electrophoretically transferred to nitrocellulose filters (2 h at 400 mV). The filters were then incubated with anti-phosphotyrosine antibody, followed by the appropriate biotinylated second antibody and developed using an enhanced chemiluminescent detection system (ECL, Amersham Pharmacia Biotech).

Activity assay of the immunoprecipitated tyrosine kinases Lyn and Syk

Control and treated cells (90 x 10⁶) were suspended for 1 h at 4°C in 450 μL of buffer containing 20 mM Tris-HCl, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, 50 mM NaCl, 1 mM Na-orthovanadate, protease inhibitor cocktail, pH 7.5. After centrifugation, the supernatants were incubated for 5 h at 4°C with either anti-Lyn or anti-Syk antibody bound to protein A-sepharose. The immuno-complexes were washed three times by centrifugation and re-suspension in 50 mM Tris/HCl, pH 7.5, containing protease inhibitor cocktail and 1 mM Na-orthovanadate. Protein tyrosine kinase (PTK) assays of immuno-complexes were performed in 30 μL of phosphorylation medium containing 50 mM Tris-HCl, 10 mM MnCl₂, pH 7.5, 50 μM γ³²P]ATP (specific activity 2000 dpm/pmol) and 100 μM Na-orthovanadate. α-synuclein (30 nM) and cdc2(6-20) peptide (200 μM) were added as substrates for Syk and Lyn, respectively (23, 25). Following incubation for 5 min at 30°C, samples were subjected to SDS-PAGE (15% gels) and substrate phosphorylation was evaluated by means of a Packard Instant Imager.

**Figure 1:** Effect of EGCG on aggregation of human platelets induced by various agonists. Panel A): at the arrows the agonists 20 U/L thrombin or 12 μM thrombin analogue peptide (TRAP) were added to platelet suspensions pre-treated with vehicle or with 25 μM EGCG. Traces are representative of five separate experiments. Panel B): effect of EGCG (25 μM) pre-incubation time on aggregation induced by thrombin (20 U/L), TRAP (12 μM), U46619 (1 μM), AlF₄⁻ (obtained by mixing, immediately before use, 15 mM NaF with 5 μM AlCl₃). Data are means of four experiments (with S.D. indicated by vertical bars), and represent the percentage of aggregation measured six min after addition of the agonists, considering 100% the value obtained in the absence of the flavanol. Panel C): solutions of thrombin (2000 U/L) or TRAP (1.2 mM) were pre-incubated with 25 μM EGCG; aliquots (2.5 μl) of the mixture were then added to the platelet suspension (250 μl), in order to give the same final agonist concentration as in A and B and a very low amount of co-added EGCG (0.25 μM), which does not affect the aggregation. Statistical significance of the differences of EGCG-treated versus untreated cells: *p < 0.05, **p < 0.01, ***p < 0.001.
**Assay of autophosphorylation and exogenous activity of purified tyrosine kinases Lyn and Syk**

Lyn and Syk tyrosine kinases were purified to apparent homogeneity from rat spleen as previously described by means of ionic exchange, gel-filtration and affinity chromatography columns including DEAE-sepharose, heparin-sepharose, polylysine-agarose and monoQ HR/FPLC for Lyn (26), and DEAE-sepharose, heparin-sepharose, phosphocellulose, super-dex-75/FPLC and monoQ HR/FPLC for Syk (27, 28). The enzyme autophosphorylation activity was tested in the above described phosphorylation medium containing 40 nM Lyn or Syk. After 10 min incubation the samples were subjected to SDS-PAGE (10% gels) and autophosphorylation was evaluated by means of a Packard Instant Imager. Exogenous phosphorylation was tested adding 200 µM cdc2(6-20) or HS1(388-402) peptides as substrates for Lyn and Syk, respectively. The incubations were halted after 10 min by addition of 1 M HCl and processed as described elsewhere (29).

**Animal treatment**

Male Wistar rats were purchased from Charles River (Calco, Lecco, Italy) and acclimatized for 1 week before treatment. They had free access to a commercial pellet diet, and were divided into three groups of five animals: one (a) had as the only accessible beverage plain water, while (b) and (c) had free access to green tea solution as unique beverage. This was prepared – using a decaffeinated green tea extract (GTE) – at concentrations 1x (b) and 4x (c) relative to the EGCG reported in a standard cup of tea (30), thus containing 1.5 and 6 mg/mL of catechins, i.e. about 0.7 mg and 2.8 mg/mL of EGCG. The animals drank about the same amount of beverage (40-50 mL per day), independently of the presence and concentration of the extract, and after one week of treatment all animals showed regular weight gain. Blood samples were obtained under anes-
thesia from the femoral vein, and treated with anticoagulant as for human plasma.

**Statistical analysis**

The statistical significance of the difference between data obtained with control and treated samples was calculated by the unpaired, two-tail t test.

**Results**

Platelet aggregation induced by thrombin and by the non-protelytic thrombin receptor activating peptide (TRAP) is reported in figure 1A. Addition of (-)epigallocatechin-3-gallate (EGCG) a few minutes prior to the agonists inhibits the process induced by thrombin more strongly than that induced by TRAP. In both cases, the inhibitory effect depends, up to about six minutes, on the time of platelet exposure to EGCG prior to the addition of the agonist (Fig. 1B). EGCG also inhibits the platelet aggregation elicited by the receptor-interacting thromboxane mimetic U46619, as well as by fluorooaluminate (AlF₄⁻), which interacts directly with G proteins (13). On the contrary, EGCG negligibly affects the aggregation induced by compounds which act downstream of the G protein activation, i.e. the calcium ionophore ionomycin (0.5 µM) and PKC activator phorbol ester (PMA, 40 nM) (not shown). A dramatic and rapid inhibitory effect on aggregation is observed if thrombin is pre-incubated with EGCG prior to its addition to the platelet suspension, whereas only a negligible effect is observed upon TRAP pre-incubation with EGCG (Fig. 1C). These results, on the one hand, are consistent with the previous observation that the flavanol inhibits the thrombin-protolytic activity (22) and, on the other, indicate that EGCG inhibits the activation cascade downstream of G-protein activation.

In agreement with this hypothesis, the catechin reduces the increase in [Ca²⁺]ᵢ elicited by thrombin and, to a lesser extent, that induced by TRAP (Fig. 2A), U46619 or fluorooaluminate (not shown). In contrast, EGCG does not appreciably affect the [Ca²⁺]ᵢ increase induced by the ionophore ionomycin. This finding rules out the possibility that the above observed inhibitory effects on calcium rise and aggregation were due to a decrease of [Ca²⁺]ᵢ consequent to the Ca²⁺-chelating property of the compound (31). EGCG also reduces the [Ca²⁺]ᵢ increase induced by thapsigargin, which, by inhibiting the endoplasmic reticulum ATPase, induces a Ca²⁺ efflux from the endoplasmic reticulum, and in the presence of extra-cellular Ca²⁺, triggers a cellular massive influx of the ion (5, 32).

The EGCG inhibitory effect on [Ca²⁺]ᵢ increase is also observed in platelets stimulated with thrombin or TRAP in the presence of the Ca²⁺ chelator ethylene-glycoltetraacetic acid (EGTA), where the [Ca²⁺]ᵢ rise is due only to IP₃-promoted Ca²⁺ efflux from the endoplasmic reticulum (Fig. 2B). However, EGCG does not affect the thapsigargin-evoked transient increase in the [Ca²⁺]ᵢ obtained in the absence of extra-cellular calcium (Fig. 2B). The dose-dependent inhibitory effect of EGCG (added to the platelet suspension prior to agonists) on

**Figure 4:** Effect of EGCG on protein phosphorylation. Panel A: autoradiographs of labeled proteins from [³²P] loaded platelets stimulated for 1 min with 20 U/L thrombin, 12 µM TRAP, or 40 nM PMA in absence or presence of 25 µM EGCG (pre-incubated for 3 min). Autoradiographs are representative of three separate experiments. Panel B: Western blotting for P-Tyr-proteins of human platelet lysates: the cells were stimulated for 1 min with thrombin (20 U/L), or TRAP (12 µM), in absence or presence of 25 µM EGCG (added 3 min prior to agonists), and treated as described in the Methods section. The figure is representative of four separate experiments.
[Ca\(^{2+}\)]\(_c\) increase is reported in figure 3, and the deduced IC\(_{50}\) are 22 and 51 \(\mu\)M versus thrombin and TRAP, respectively. Similar results are also obtained for platelet aggregation (not shown). EGCG pre-incubated with thrombin limits the agonist-induced [Ca\(^{2+}\)]\(_c\) increase much more effectively than when added to platelet suspension; in fact under these conditions the calculated IC\(_{50}\) is about 7 \(\mu\)M. This value is about one order of magnitude less than that determined for synthetic substrate degradation (22). By contrast, EGCG pre-incubated with TRAP does not affect the [Ca\(^{2+}\)]\(_c\) increase induced by this agonist.

Platelet activation involves the Ser/Thr phosphorylation of pleckstrin (47 kDa), which is one of the most important PKC-dependent phosphorylating events associated with this process (14). Pleckstrin phosphorylation is decreased by the presence of EGCG in suspensions of platelets stimulated with thrombin and - to a lesser extent - with TRAP, whereas it is not changed in platelets stimulated with the specific PKC activator PMA (Fig. 4A).

Platelet stimulation by various agonists is also accompanied by a remarkable increase in Tyr phosphorylation of many cellular proteins (6, 7, 33-37). Fig. 4B shows the pattern of platelet protein Tyr phosphorylation induced by thrombin or TRAP and the inhibitory effect of EGCG added to platelet suspensions. The inhibition is particularly evident on the thrombin-induced phosphorylation of the protein bands of about 60, 72 and 125 kDa. Since PTKs belonging to the Src family (55-62 kDa) and Syk (72 kDa) are included in the group of enzymes whose autophosphorylation is associated with platelet activation, and positively correlates with their activity (34), we tested, and found notably increased, the kinase activity of the anti-Syk and anti-Lyn immuno-precipitated complexes obtained from stimulated platelets. EGCG strongly counteracts the increase of activity – tested on specific substrates – of both immuno-complexes obtained from thrombin-stimulated cells and to a lesser extent, that evoked by TRAP (Figs. 5A and 5B). A direct and dose-dependent inhibitory effect of EGCG is found on the in vitro autophosphorylation and kinase activity of Lyn and Syk, purified from rat spleen, and tested toward the specific substrates cdc2(6-20) and HS1(388-402) peptides, respectively (26, 28). The flavanol inhibits the kinase activity of Lyn and Syk with IC\(_{50}\) values of 3.1 and 2.2 \(\mu\)M; and their autophosphorylation with IC\(_{50}\) of 1.8 and 9.7 \(\mu\)M, respectively (Fig. 5C).

Based on these results, we next investigated whether oral administration of green tea solutions to rats affected their platelet activation. We first tested the effect of EGCG on acti-
vation of platelets obtained from control rats and found that, as in the case of human cells, the flavanol strongly inhibits thrombin-induced aggregation and [Ca\textsuperscript{2+}]\textsubscript{c} increase (not shown). As TRAP is unable to activate rat platelets (38), it was obviously not included in these experiments.

We then examined the activation of platelets obtained from rats given green tea solution as unique beverage. Figure 6 shows that both thrombin-induced aggregation and [Ca\textsuperscript{2+}]\textsubscript{c} increase are reduced, in a dose-dependent manner, in platelets obtained from rats given green tea solutions compared with those from control animals; indicating that the GTE inhibitory effect persists in platelets no longer exposed to catechins.

**Discussion**

We have recently shown that (-)epigallocatechin-3-gallate, the major flavanol contained in green tea, is a good inhibitor of leukocyte elastase – the potent proteolytic weapon used by neutrophils at the site of inflammation – and, although less efficiently, it hinders the activity of cathepsin G, urokinase plasminogen activator as well as the *in vitro* thrombin proteolytic activity on a synthetic substrate and gelatin (22). We demonstrate here that EGCG inhibits thrombin-elicited human platelet aggregation and the cytosolic calcium increase more strongly than those induced by non-proteolytic TRAP, which activates platelets by interaction with the same receptor of thrombin but without cleaving it. Moreover the flavanol, pre-incubated with the agonist prior to addition to platelet suspension, inhibits the activation induced by thrombin but not by TRAP. This evidence supports the conclusion that EGCG hinders the thrombin proteolytic activity.

EGCG also reduces the [Ca\textsuperscript{2+}]\textsubscript{c} increase induced by both thrombin and TRAP in the absence of extra-cellular calcium, but does not change either the thapsigargin-evoked calcium efflux from endoplasmic reticulum or the ionomycin-induced influx of extra-cellular calcium (and ensuing aggregation). Together these results suggest that the flavanol exerts its inhibition not only on thrombin-mediated cleavage of PAR(s) but also on a metabolic process involved in Ca\textsuperscript{2+} efflux from endoplasmic reticulum. It has been previously reported that green tea catechins inhibit calcium increase and platelet aggregation – induced in platelet-rich plasma – by ADP, collagen or epinephrine (16, 17). However at variance with Kang’s report (16), which shows an inhibitory effect of EGCG also on aggregation induced by the calcium ionophore A23187, we found no significant inhibitory effect of EGCG on this process elicited by the ionophore ionomycin. This discrepancy is probably due to the different experimental conditions employed: in fact, Kang’s group measured the platelet aggregation in platelet-rich plasma using much higher concentrations of catechins than those used here with washed platelet suspensions. This group also reported that EGCG and other green tea catechins (GTC) inhibit the

*ex vivo* aggregation of platelet rich plasma obtained from rats administered a high dose of green tea extract solutions (100 mg/kg) by gastric tube. Nonetheless, thrombin, pro-thrombin, and activated partial thromboplastin times remained unchanged, leaving the mode of EGCG anti-thrombotic action undetermined. It should also be stressed that we registered GTC-exerted inhibition also in platelets isolated from rat blood, and therefore no longer exposed to flavanol. This finding, consistently with the exposure-time-dependent EGCG inhibitory...
effects observed on platelet activation evoked by non-proteolytic agonists, indicates that EGCG is incorporated by platelets. We have indeed confirmed this conclusion (manuscript in preparation), which is in agreement with a recent report showing that EGCG is taken up by adenocarcinoma cells (39).

To shed further light on the signal transduction cascade steps specifically affected by EGCG, we analyzed the protein Ser-, Thr-, and Tyr-phosphorylation triggered by thrombin and TRAP. As the PKC-dependent pleckstrin phosphorylation – similarly to aggregation - is inhibited by EGCG in platelets stimulated with thrombin and TRAP but not in those stimulated with PMA, we conclude that the flavanol does not affect this enzyme and that the reduced pleckstrin phosphorylation, observed in cells stimulated with thrombin or TRAP, is probably due to a reduced increase in Ca\(^{2+}\) and diacylglycerols, which are well known PLC-products activating PKC (14).

EGCG strongly counteracts the protein Tyr-phosphorylation triggered by thrombin and to a lesser extent by TRAP, in particular that of proteins displaying apparent Mr of 60, 72, and 125 kDa. More than 60 proteins were found to be unique in the thrombin-activated platelet proteome when compared to resting platelets (36), and few of them have been identified – including PTKs belonging to the Src family (55-62 kDa), Syk (72 kDa) and Fak (125 kDa) (34-37), which show a Mr in the range corresponding to those of the main targets of EGCG inhibition. Here we demonstrate, by immuno-precipitation experiments with specific antibodies, that thrombin-induced Tyr-autophosphorylation and the parallel activation of Syk and Src-related Lyn are strongly inhibited by EGCG. In addition, we identified these two non-receptor Tyr kinases as direct targets of the flavanol, which inhibits the autophosphorylation and enzyme activity of purified enzymes with relatively low IC\(_{50}\) values.

It has been reported that EGCG inhibits the focal adhesion kinase FAK (40), as well as the Tyr-phosphorylation of various receptors including EGF-R of carcinoma cells, and PDGF-Rbeta of human glioblastoma, and vascular cells (40-43). Our data show that, in stimulated platelets, EGCG inhibits the non-receptor PTKs Lyn and Src and [Ca\(^{2+}\)]\(_c\) increase. The definition of the hierarchy between these two events represents an intriguing matter not yet completely resolved, mainly because protein Tyr-phosphorylation occurs in different temporal waves (7), and cytosolic calcium derives from different sources (32). In fact it has been demonstrated, on the one hand, that in platelets, as in other non-excitatory cells, [Ca\(^{2+}\)]\(_c\) increase triggers the tyrosine phosphorylation of multiple proteins (6,7,44-47). On the other hand, activation of PTKs may occur through a Ca-independent mechanism and may constitute a process which regulates [Ca\(^{2+}\)]\(_c\) rise – originating from both endoplasmic reticulum and extra-cellular compartment – by activation of PLC-\(\gamma\) and IP\(_3\) kinase and the “capacitative” Ca\(^{2+}\) influx, respectively (43, 48-53).

In conclusion, these results indicate that EGCG displays a multiple effect on platelet activation as it inhibits both the thrombin-mediated proteolytic cleavage of PAR(s), and the activity of the Tyr-kinases Syk and Lyn, which are most probably involved in the regulation of [Ca\(^{2+}\)]\(_c\) increase evoked by both thrombin and non-proteolytic agonists. It is well documented that catechins have powerful antioxidant as well as reactive oxygen species scavenging properties (21, 54, 55). Whether the actions shown by EGCG on platelet activation are due to the antioxidant properties of the compound or to other factors deserves further investigation. However, these results indicate that the green tea catechin EGCG is a potent anticoagulant compound with potential cardiovascular beneficial properties.

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