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Ethylene Glycol Bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic Acid (EGTA) and the Tyrphostin ST271 Inhibit Phospholipase C in Human Platelets by Preventing Ca^{2+} Entry

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SUMMARY

In the present study, the roles of Ca^{2+} and fibrinogen receptor occupancy in the regulation of phospholipase C by G protein-coupled and tyrosine kinase-linked receptor pathways in human platelets have been investigated. Agonist stimulation of phospholipase C was not altered significantly in the absence of stirring or in the presence of the fibrinogen receptor antagonist arginine-glycine-aspartate-serine, conditions that prevent platelet aggregation. Similarly, elevation of intracellular Ca^{2+} levels by the ionophores A23187 or ionomycin did not induce formation of inositol phosphates. In contrast, chelation of extracellular Ca^{2+} by ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) reduced formation of inositol phosphates by G protein receptor (thrombin)- and tyrosine kinase (Fc receptor and peroxovanadate)-regulated pathways. Similarly, short term exposure to Ni^{2+} ions, which also prevent Ca^{2+} entry, inhibited thrombin-stimulated formation of inositol phosphates. Loading of platelets with the intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) markedly suppressed elevation of intracellular Ca^{2+} and formation of inositol phosphates in platelets stimulated by G protein receptor- and tyrosine kinase-regulated pathways. The greater inhibition of phospholipase C by BAPTA, relative to that induced by EGTA, is consistent with the more pronounced inhibition of intracellular Ca^{2+} elevation. The tyrphostin tyrosine kinase inhibitor ST271 also reduced intracellular Ca^{2+} levels and inhibited activation of phospholipase C. The degree of inhibition of phospholipase C by ST271 was slightly greater than that induced by EGTA but was not additive with the effect of EGTA, suggesting a common mode of action. It is concluded that elevation of intracellular Ca^{2+} regulates agonist-induced activation of phospholipase C and that this contributes to the inhibition of thrombin-induced formation of inositol phosphates by the tyrphostin ST271.

A wide variety of extracellular stimuli induce activation of human platelets through hydrolysis of phosphoinositides, generating the second messengers inositol-1,4,5-trisphosphate and diacylglycerol (1). Increasing evidence suggests that this second messenger pathway is regulated in platelets through two distinct mechanisms, involving G proteins or tyrosine kinases. Many platelet receptors belong to the seven-transmembrane domain class of proteins that mediate their effects through G proteins, including thrombin and thromboxane A_2 receptors (2, 3). It is well established that G protein-coupled receptors induce activation of phospholipase C-β isoforms through generation of α subunits of the $G_{q/11}$ family of G proteins and liberation of βγ subunits (4, 5). On the other hand, receptors for stimuli that induce clustering of membrane proteins on the platelet surface, e.g., collagen and immune complexes (which activate the platelet FcγRII) (6), lack the seven-transmembrane domain structure characteristic of G protein-coupled receptors, and increasing evidence suggests that they mediate their effects through tyrosine phosphorylation of phospholipase C-γ_2 (7, 8). In support of this, activation of phospholipase C by collagen and immune complexes is completely inhibited by the nonselective serine/threonine kinase and tyrosine kinase inhibitor staurosporine and by the more selective tyrphostin tyrosine kinase inhibitor ST271 (9–11). In contrast, staurosporine has no significant effect on the activation of phospholipase C in platelets by thrombin or U46619 (a thromboxane mimetic), although ST271 induces partial inhibition of this response (11, 12).

The inhibitory effect of ST271 against thrombin and U46619 provides evidence that G protein-coupled receptors may also induce activation of phospholipase C in part through a tyrosine kinase-dependent pathway. However, this is not supported by the lack of effect of staurosporine or the absence of tyrosine phosphorylation of phospholipase C-γ_2 in

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ABBREVIATIONS: FcγRII, FcγIIA receptor; RGDS, arginine-glycine-aspartate-serine; ST271, α-cyano-4-hydroxy-3,5-diisopropylcinnamide; U46619, 11α,9α-epoxymethano-prostaglandin H_2; PtdIns4,5P_2, phosphatidylinositol-4,5-bisphosphate; AM, acetoxymethyl ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mAb, monoclonal antibody; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

thrombin- and U46619-stimulated platelets (8). Moreover, caution is required in the use of tyrphostins, because at the relatively high concentrations required to inhibit tyrosine phosphorylation they induce nonspecific effects, including inhibition of oxidative phosphorylation (13).

Despite the clear evidence for two distinct pathways of regulation of phosphoinositide metabolism in platelets, it is unclear whether other events also contribute to the regulation of phospholipase C. For example, the role of intracellular Ca^{2+} in the regulation of phospholipase C is unclear. Rittenhouse (14) and Lapetina *et al* (15) reported that the Ca^{2+} ionophore A23187 does not induce activation of phospholipase C in human platelets in the presence of inhibitors of cyclooxygenase. On the other hand, Siess and Lapetina (16) observed a small increase in formation of phosphatidic acid produced by A23187, which may be derived by activation of phospholipase C. Similarly, the role of the fibrinogen receptor in the regulation of phospholipase C is unclear. Sinigaglia *et al* (17) observed decreased formation of inositol phosphates in platelets stimulated by low concentrations of thrombin in the presence of fibrinogen or an arginine-glycine-aspartate-containing peptide that binds to the fibrinogen receptor; however, a similar effect was not observed with higher concentrations of thrombin. Activation of the fibrinogen receptor increases tyrosine phosphorylation of a range of proteins in platelets (18, 19), although it is not known whether this includes the phospholipase C-γ isoform.

The aim of the present study was to investigate further the role of the fibrinogen receptor and intracellular Ca^{2+} in the regulation of phospholipase C by G protein-coupled and tyrosine kinase-linked receptors. The results demonstrate a requirement for Ca^{2+} in the formation of inositol phosphates by both sets of stimuli but provide evidence against a role of fibrinogen receptor occupancy or platelet aggregation in this response. Inhibition of thrombin-induced phospholipase C activation by the tyrphostin ST271 may be mediated, at least in part, through inhibition of Ca^{2+} entry.

Experimental Procedures

Materials. Thrombin, the peptide RGDS, indomethacin, phorbol dibutyrate, and sheep $F(ab')_2$ raised against mouse IgG (M-1522) were from Sigma (Poole, Dorset, UK). Collagen (native collagen fibrils from equine tendons) was from Nycomed (Munich, Germany). mAb IV.3 (specific for FcγRII) was from Madarex (Annandale, NJ). BAPTA/AM and fura-2/AM were from Calbiochem-Novabiochem (Nottingham, UK). *myo*-[^{3}H]Inositol (specific activity, 18.2 Ci/mmol) was from Amersham (Cardiff, UK). ST271 was a gift from the Wellcome Foundation (Beckenham, UK). All other reagents were of analytical grade. Stock solutions of BAPTA/AM and indomethacin were dissolved in dimethylsulfoxide; the volume of dimethylsulfoxide added to experimental samples did not exceed 0.2% and was present in controls.

Platelet preparation and measurement of [^{3}H]inositol phosphates. On the day of the experiment blood was drawn from drug-free volunteers, using sterile 20 mM sodium citrate as anticoagulant. Platelet-rich plasma was obtained by centrifugation at 200 × *g* for 20 min. Platelets were isolated from platelet-rich plasma by centrifugation at 1000 × *g* for 10 min in the presence of prostacyclin (0.1 μg/ml) to elevate intracellular cAMP. Platelets were resuspended at 37° in 1 ml of a modified Tyrodes-HEPES buffer (138 mM NaCl, 0.36 mM NaH_2PO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3) and labeled with [^{3}H]inositol (50 μCi/ml) for 3 hr. Platelets were then centrifuged in the presence of prostacyclin (0.1 μg/ml) at 1000 × *g* for 10 min and resuspended at a concentration of 2–4 × 10^8/ml in the aforementioned buffer containing LiCl (10 mM) and indomethacin (10 μM). Platelets were left at room temperature for at least 30 min before experimentation. Platelet suspensions (0.24 ml) were incubated at 37° for 15 min, in the absence or in the presence of BAPTA/AM, before transfer to a Chrono-log aggregometer, where they were stirred continuously at 1200 rpm for the remainder of the experiment (unless stated otherwise). RGDS, ST271, or EGTA was added 60 sec after transfer to the aggregometer and incubated for 3 min before addition of thrombin or collagen. Activation of FcγRII was achieved by cross-linking with mAb IV.3 (1 μg/ml) and $F(ab')_2$ anti-mouse IgG (30 μg/ml); mAb IV.3 was added 60 sec before $F(ab')_2$ addition, with the latter time being taken as the start of stimulation. Peroxovanadate was made by addition of orthovanadate (400 μM) to the platelet sample 60 sec before addition of 2 mM hydrogen peroxide (9), with the latter time being taken as the start of stimulation. All experiments were stopped after 5 min (unless stated otherwise) by transfer to 940 μl of chloroform/methanol/HCl (50:100:1); water (0.31 ml) and $CHCl_3$ (0.31 ml) were then added to enable phase separation. Total [^{3}H]inositol phosphates (i.e., inositol mono-, bis-, and trisphosphates) were separated from [^{3}H]inositol by Dowex anion exchange chromatography as described previously (12).

Measurement of intracellular Ca^{2+} levels. Platelet-rich plasma was incubated with fura-2/AM (1.5 μM) at 30° for 45 min, followed by sedimentation and resuspension in Tyrode-HEPES buffer containing 10 μM indomethacin, to a cell density of 3 × 10^8/ml. Fluorescence intensities were measured at fluorescence excitation wavelengths of 340 nm and 380 nm, with emission at 510 nm, using a Perkin-Elmer LS50B spectrofluorimeter; data are presented as the fluorescence ratio (340/380 nm). Experiments were conducted at room temperature or 37°. Experimental traces were corrected for ST271 absorbance by normalization to basal fluorescence before addition of the tyrphostin.

Analysis of results. Measurements of [^{3}H]inositol phosphates were performed in quadruplicate. Results are shown either as data from a single experiment representative of two others or as pooled data from several experiments. In the latter case, data were normalized to the response induced by 1 unit/ml thrombin; this was essential because of variation between experiments in the basal level of [^{3}H]inositol phosphates and maximal increases observed. Statistical analyses were performed using Student's *t* test, with $p < 0.05$ being taken as the level of significance.

Results

EGTA inhibition of agonist-induced formation of inositol phosphates. Agonist stimulation of phospholipase C was investigated by measurement of [^{3}H]inositol phosphates in the presence of 10 mM LiCl, which inhibits metabolism of inositol monophosphates to free inositol. Under these conditions, a decrease in the level of [^{3}H]inositol phosphates is the consequence of reduced formation rather than enhanced metabolism.

The role of platelet aggregation and fibrinogen receptor occupancy was investigated using three different sets of conditions, as follows: (i) platelets do not undergo aggregation in the absence of stirring when present at a density of $<1 \times 10^9$/ml; (ii) the small peptide RGDS, which contains the binding motif characteristic of integrin receptors, binds to the fibrinogen receptor (glycoprotein IIb-IIIa), preventing aggregation; and (iii) the glycoprotein IIb-IIIa complex is destabilized when the extracellular Ca^{2+} concentration is reduced to <1 nM in the presence of 1 mM EGTA. Fig. 1 illustrates that neither the absence of stirring nor the presence of RGDS altered thrombin (0.1 unit/ml)- or FcγRII-induced formation

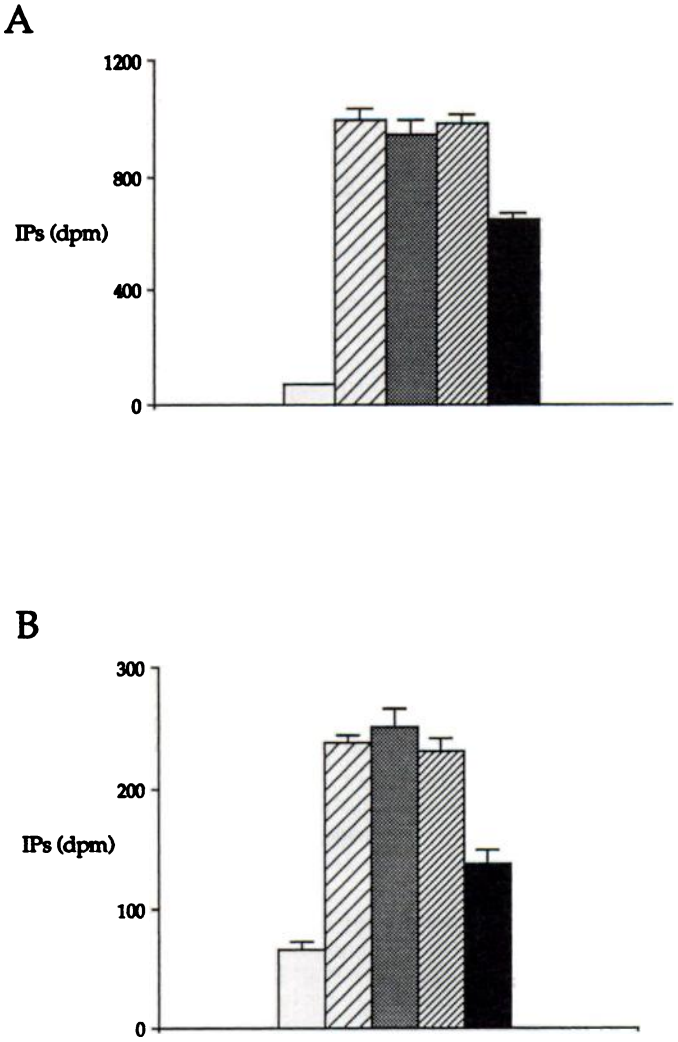

Fig. 1. Role of aggregation in agonist-induced formation of [^{3}H]inositol phosphates. Human platelets were labeled with [^{3}H]inositol, as described in Experimental Procedures, and stimulated by thrombin (0.1 unit/ml) (A) or cross-linking of the FcγRII [1 μg/ml mAb IV.3 and 30 μg/ml F(ab')$_2$] (B) for 5 min in the presence of LiCl (10 mM). Formation of total [^{3}H]inositol phosphates (*IPs*) was measured by Dowex anion exchange chromatography. Results are shown as basal inositol phosphate levels in stirred platelets (□) or levels in agonist-stimulated cells treated as follows: stirring at 1200 rpm (▨), no stirring (■), stirring at 1200 rpm in the presence of 100 μg/ml RGDS (■), or stirring at 1200 rpm in the presence of 1 mM EGTA (■). Results are shown as mean ± standard error of one experiment performed in quadruplicate, which is representative of two others. For some points, the standard error is hidden by the symbol.

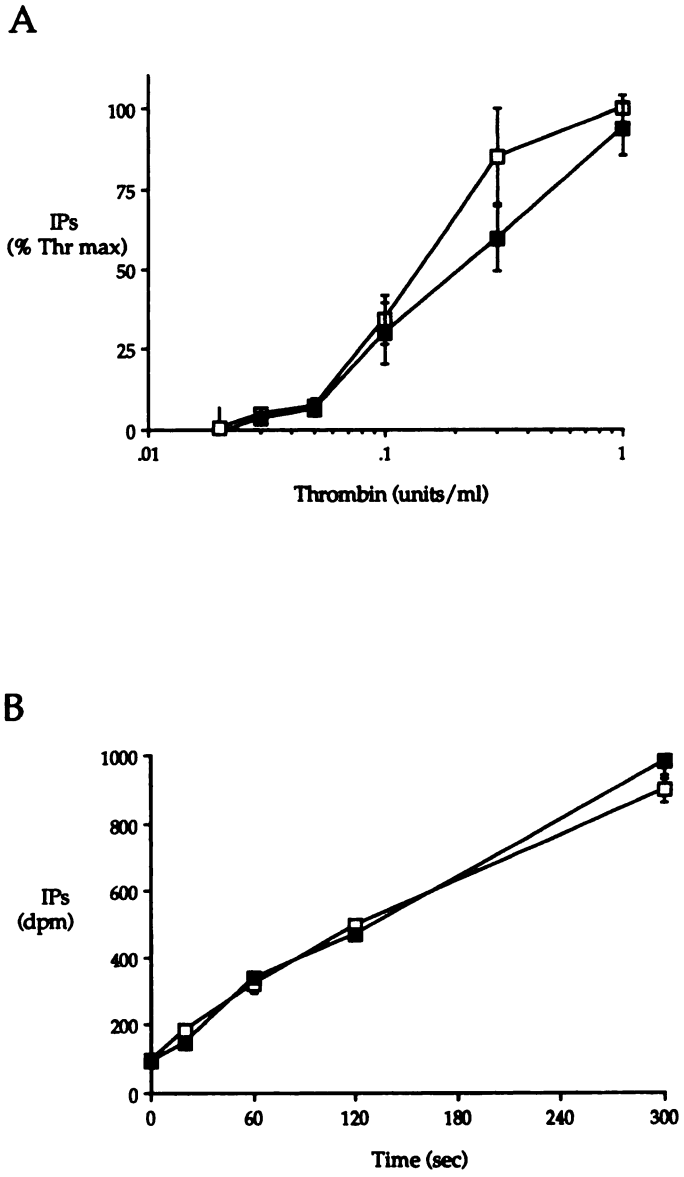

Fig. 2. Effect of RGDS on the concentration-response curve and time course of thrombin-induced formation of [^{3}H]inositol phosphates. Human platelets were labeled with [^{3}H]inositol as described in Experimental Procedures. Formation of total [^{3}H]inositol phosphates (*IPs*) was measured by Dowex anion exchange chromatography. A, Concentration-response curve (5-min incubation) for thrombin (*Thr*)-induced formation of inositol phosphates in the absence (□) and presence (■) of 100 μg/ml RGDS. B, Time course of thrombin (0.1 unit/ml)-induced formation of inositol phosphates in the absence (□) and presence (■) of 100 μg/ml RGDS. Results shown in A represent mean ± standard error from four separate experiments performed in quadruplicate, whereas those in B are from a single experiment performed in quadruplicate. Results similar to those shown in B were observed in two other experiments and in an additional experiment using 1 unit/ml thrombin. For some points, the standard error is hidden by the symbol.

of [^{3}H]inositol phosphates. In view of the previous report by Sinigaglia *et al* (17) that fibrinogen receptor occupancy modifies the response to low but not high concentrations of thrombin, the effect of RGDS was studied over a range of concentrations of thrombin. Fig. 2A demonstrates that RGDS had no significant effect on the concentration-response curve for thrombin-induced formation of [^{3}H]inositol phosphates. Similarly, RGDS did not alter the time course of thrombin-induced formation of [^{3}H]inositol phosphates (Fig. 2B).

In marked contrast to the results observed in the absence of stirring or in the presence of RGDS, the Ca^{2+} chelator EGTA reduced both thrombin- and FcγRII-induced formation of [^{3}H]inositol phosphates by ~40% (Fig. 1). This effect could be overcome by the addition of extracellular Ca^{2+} (2 mM), demonstrating that it is not due to a direct effect of EGTA but is more likely the result of removal of extracellular Ca^{2+}. The inhibitory action of EGTA occurs over the length of the concentration-response curve for thrombin (Fig. 3A) and appears to be delayed in onset, in that EGTA had no significant effect on the initial formation of [^{3}H]inositol phosphates stimulated by thrombin (Fig. 3B). A similar inhibitory effect of 1 mM

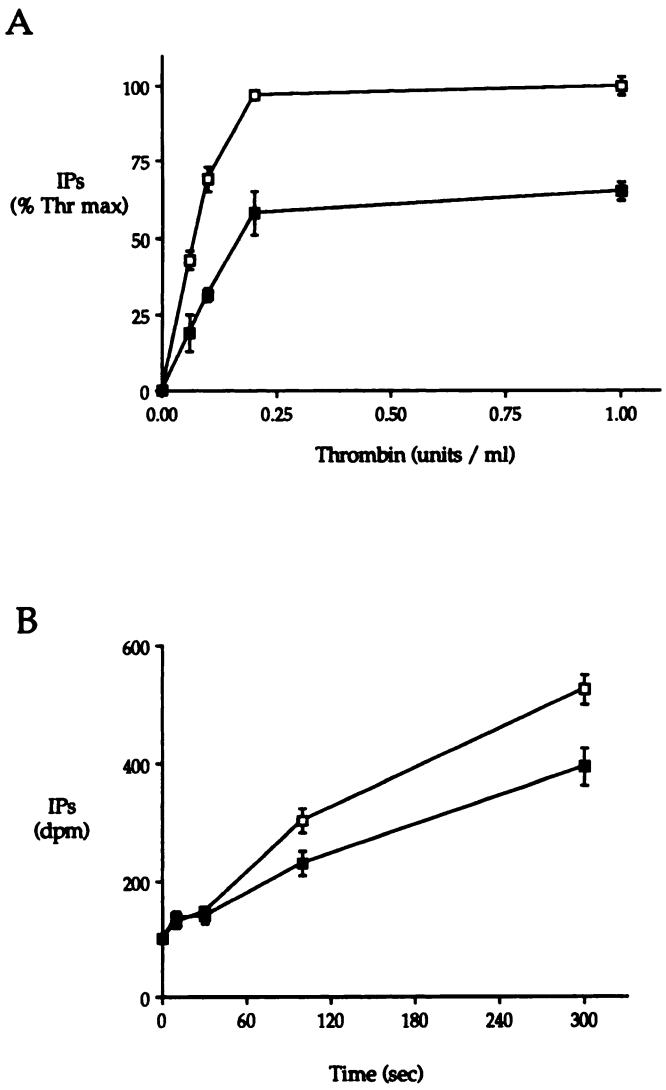

Fig. 3. Effect of EGTA on the concentration-response curve and time course of thrombin-induced formation of [^{3}H]inositol phosphates. Human platelets were labeled with [^{3}H]inositol as described in Experimental Procedures. Formation of total [^{3}H]inositol phosphates (*IPs*) was measured by Dowex anion exchange chromatography. A, Concentration-response curve (5-min incubation) for thrombin (*Thr*)-induced formation of inositol phosphates in the absence (□) and presence (■) of 1 mM EGTA. B, Time course of thrombin (1 unit/ml)-induced formation of inositol phosphates in the absence (□) and presence (■) of 1 mM EGTA. Results shown in A represent mean ± standard error from three separate experiments performed in quadruplicate, whereas those in B are from a single experiment performed in quadruplicate. Results similar to those shown in B were observed in two other experiments. For some points, the standard error is hidden by the symbol.

EGTA was observed against the tyrosine phosphatase inhibitor peroxovanadate (response in the presence of EGTA, 71.1 ± 2.4% of controls), which induces activation of phospholipase C-γ isoforms in platelets through tyrosine phosphorylation (8, 9).

BAPTA/AM inhibition of agonist-induced formation of inositol phosphates. The inhibitory effect of EGTA indicates that extracellular Ca^{2+} or increased intracellular Ca^{2+} levels may play a role in agonist-induced formation of [^{3}H]inositol phosphates. To investigate this further, increases in intracellular Ca^{2+} were buffered with BAPTA/AM, which is converted in the cytosol to its free acid form (20). BAPTA free acid is unable to diffuse across plasma membranes and is therefore trapped in the cytosol, where it is able to chelate intracellular Ca^{2+}. Platelets take up BAPTA/AM over a 15-min incubation, with the final intracellular concentration reached being dependent on platelet density. Fig. 4 illustrates that BAPTA/AM markedly depressed thrombin-induced elevation of intracellular Ca^{2+} levels over the concentration range of 10–40 μM, at a platelet density of 2×10^8/ml. At the highest concentration of BAPTA/AM used (40 μM), only a small and gradual increase in intracellular Ca^{2+} levels was observed in thrombin-stimulated cells (Fig. 4).

The loading of platelets with BAPTA markedly inhibited agonist-induced formation of [^{3}H]inositol phosphates. Representative traces showing the concentration dependence of BAPTA/AM-induced inhibition of [^{3}H]inositol phosphate formation stimulated by thrombin and peroxovanadate are shown in Fig. 5. The responses to both stimuli were inhibited over the concentration range of 10–40 μM BAPTA/AM, with the level of formation of [^{3}H[inositol phosphates after incubation with 40 μM BAPTA/AM being <15% of that in controls. Similar results were seen in the absence and presence of EGTA and also for platelet activation by mAb IV.3/F(ab′)$_2$ and collagen (data not shown). Smith *et al.* (21) previously reported inhibition of collagen-induced formation of phosphatidic acid in BAPTA-loaded platelets, although the magnitude of that effect was smaller than that observed in the present study. The greater inhibitory effect of BAPTA/AM on formation of inositol phosphates, relative to EGTA, may be explained by the more marked depression of intracellular Ca^{2+} induced by the former compound (Figs. 4 and 6).

Tyrphostin ST271 reduction of thrombin-induced formation of inositol phosphates through inhibition of Ca^{2+} elevation. The tyrosine kinase inhibitors genistein and the tyrphostin methyl-2,5-dihydroxycinnamate inhibit entry of extracellular Ca^{2+} in human platelets stimulated by thrombin, ADP, or U46619 (22–24). In accord with this, ST271 inhibited thrombin-induced elevation of Ca^{2+} to an extent similar to that induced by EGTA (Fig. 6), suggesting that these compounds induce inhibition through a common mechanism. i.e., inhibition of Ca^{2+} entry. This conclusion is supported by the observation that the combined action of EGTA and ST271 is similar to the effect of either substance alone (data not shown).

The inhibition of Ca^{2+} elevation by ST271 may explain its ability to reduce thrombin-stimulated formation of [^{3}H]inositol phosphates (11). This was investigated through additivity experiments with EGTA. ST271 induced a slightly greater inhibition of formation of [^{3}H]inositol phosphates than that seen with EGTA ($p < 0.05$) (Fig. 7), consistent with the increased inhibition of intracellular Ca^{2+} elevation. When EGTA and ST271 were used together, however, the degree of inhibition observed was no greater than that seen with ST271 (Fig. 7), suggesting that ST271 mediates its effects in part through the same pathway as EGTA, i.e., the inhibition of Ca^{2+} entry.

Ni^{2+} has been also reported to inhibit entry of Ca^{2+} in platelets by blockade of the Ca^{2+} entry channel (25). Consistent with this, Ni^{2+} inhibited thrombin-induced formation of [^{3}H]inositol phosphates, with the extent of inhibition being dependent on the time of preincubation (data not shown). Addition of 2 mM Ni^{2+} 60 sec before thrombin addition reduced the formation of [^{3}H]inositol phosphates to a level

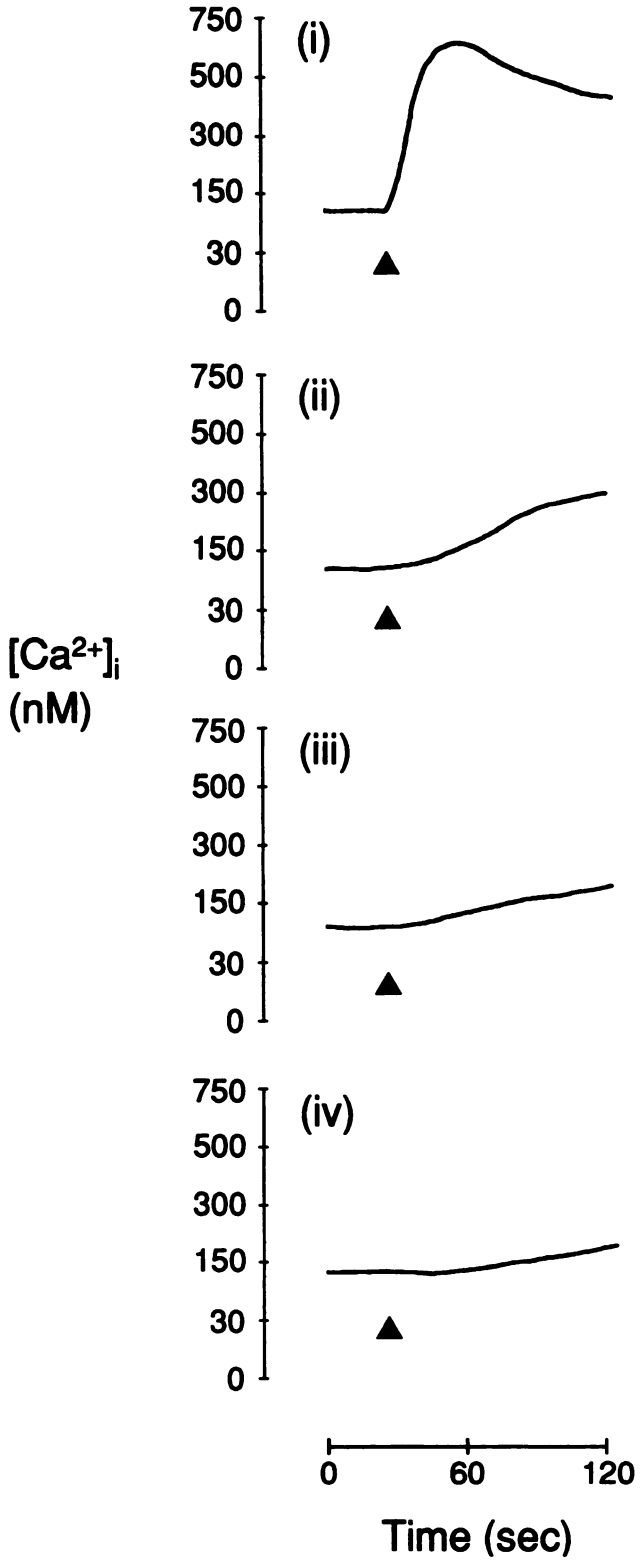

Fig. 4. BAPTA inhibition of thrombin-induced Ca^{2+} elevation in human platelets. Human platelets were loaded with fura-2, as described in Experimental Procedures, and incubated (at a density of 2×10^8/ml) with BAPTA/AM, in the presence of 1 mM EGTA, for 15 min before addition of 1 unit/ml thrombin (▲). The elevation of intracellular Ca^{2+} is reflected by the increase in the fluorescence ratio (excitation at 340 and 380 nm; emission at 510 nm), measured as described in Experimental Procedures. i, Control; ii, 10 μM BAPTA/AM; iii, 20 μM BAPTA/AM; iv, 40 μM BAPTA/AM. Results are shown as typical traces from one experiment, which is representative of one other experiment.

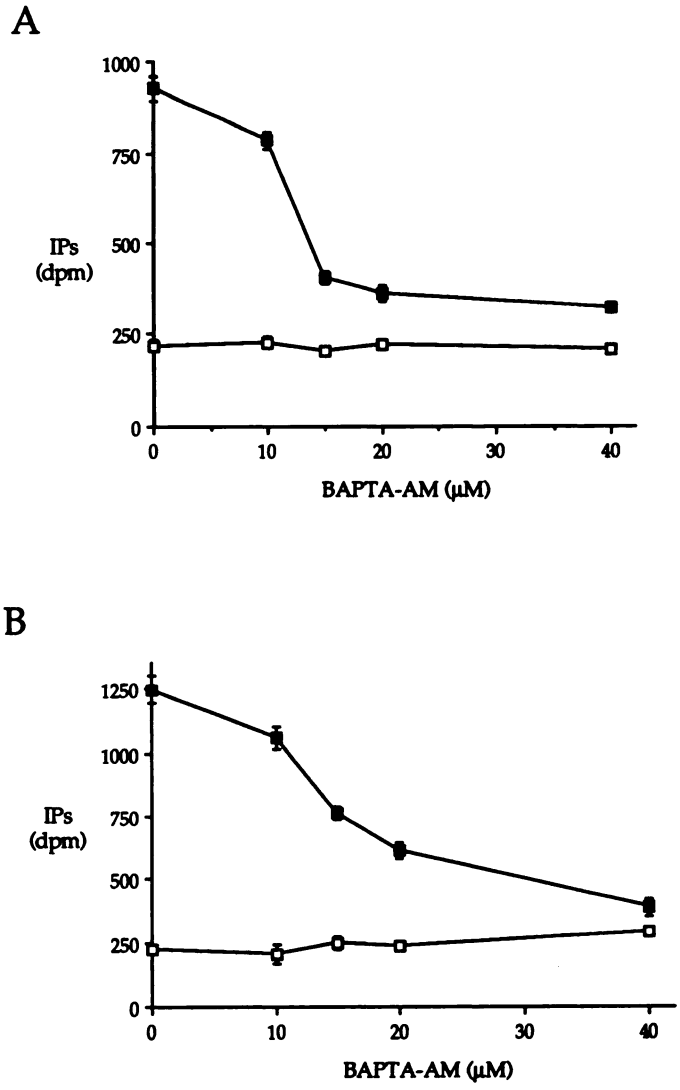

Fig. 5. Concentration-response curve for BAPTA/AM-induced inhibition of [^{3}H]inositol phosphate formation induced by thrombin and peroxovanadate. Human platelets were labeled with [^{3}H]inositol, as described in Experimental Procedures, and resuspended in buffer containing 1 mM EGTA. Formation of total [^{3}H]inositol phosphates (*IPs*) was measured, after a 5-min incubation with each stimulus, by Dowex anion exchange chromatography. Platelets (2×10^8/ml) were preincubated with BAPTA/AM for 15 min before addition of thrombin or peroxovanadate. A, Level of inositol phosphates in the absence (□) or presence (■) of 1 unit/ml thrombin; B, level of inositol phosphates in the absence (□) or presence (■) of peroxovanadate. Peroxovanadate was made from 400 μM orthovanadate and 2 mM H_2O_2, as described in Experimental Procedures. Results are shown as mean ± standard error from one representative experiment performed in quadruplicate, which is representative of two other similar experiments. For some points, the standard error is hidden by the symbol.

similar to that seen with ST271 (Fig. 7). However, when these compounds were used together, and in marked contrast to the lack of additivity observed between ST271 and EGTA, their combined effects were partially additive (Fig. 7), suggesting that part of the inhibitory effect of Ni^{2+} must occur through a pathway distinct from that used by ST271. A similar additivity experiment could not be performed with Ni^{2+} and EGTA because of formation of Ni^{2+}-EGTA complexes.

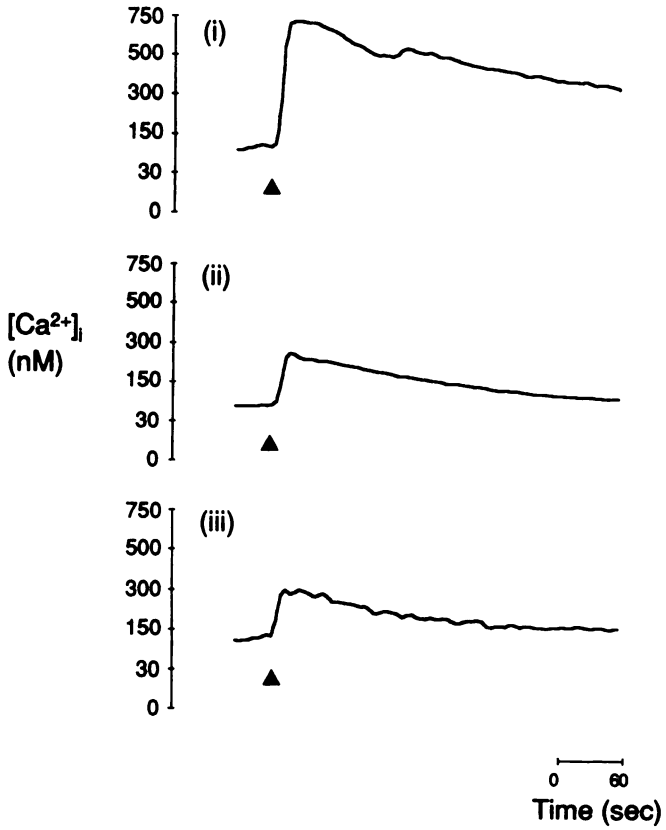

Fig. 6. Inhibition by EGTA and ST271 of thrombin-induced Ca^{2+} elevation in human platelets. Human platelets were loaded with fura-2, as described in Experimental Procedures, and challenged with 1 unit/ml thrombin (▲). The elevation of intracellular Ca^{2+} is reflected by the increase in the fluorescence ratio (excitation at 340 and 380 nm; emission at 510 nm), measured as described in Experimental Procedures. i, Control; ii, 1 mM EGTA; iii, 300 μM ST271. Results are shown as typical traces from one experiment, which is representative of three other similar experiments.

Effect of Ca^{2+} ionophores on the level of inositol phosphates. Siess and Lapetina (16) reported concentration-dependent formation of [^{32}P]phosphatidic acid induced by the Ca^{2+} ionophore A23187, with a maximal response being observed at 1 μM A23187. This result is in contradiction to earlier observations of Rittenhouse (14) and Lapetina *et al.* (15), who did not observe an increase in formation of [^{32}]phosphatidic acid in platelets challenged with A23187 (0.1–5 μM). The explanation for this discrepancy is not known. Moreover, the mechanism of the A23187-induced formation of [^{32}]phosphatidic acid observed by Siess and Lapetina (16) may be unrelated to phosphoinositide metabolism, because phosphatidic acid can be derived from a number of pathways, e.g., phospholipase D.

We measured levels of [^{3}H]inositol phosphates in the presence of 10 mM LiCl and 10 μM indomethacin in human platelets stimulated with the Ca^{2+} ionophores A23187 and ionomycin, as a direct monitor of phospholipase C activation. Neither A23187 (2 μM) nor ionomycin (1 μM) increased the level of [^{3}H]inositol phosphates, relative to controls, in the absence of added Ca^{2+}, despite marked stimulation of aggregation (Fig. 8A and data not shown). Similar results were seen in the combined presence of the phorbol ester phorbol dibutyrate and ionomycin (data not shown). The action of

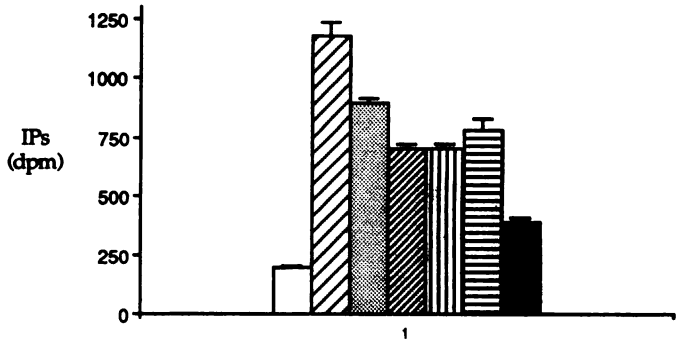

Fig. 7. Absence of additivity in EGTA and ST271 inhibition of thrombin-induced formation of [^{3}H]inositol phosphates. Human platelets were labeled with [^{3}H]inositol as described in Experimental Procedures. Formation of total [^{3}H]inositol phosphates (*IPs*) was measured, after a 5-min incubation with thrombin (1 unit/ml), by Dowex anion exchange chromatography. Formation of inositol phosphates was measured under the following conditions: control (□), thrombin (■), thrombin plus 1 mM EGTA (■), thrombin plus 300 μM ST271 (■), thrombin plus 2 mM $NiCl_2$ (■), thrombin plus 1 mM EGTA and 300 μM ST271 (■), and thrombin plus 300 μM ST271 and 2 mM $NiCl_2$ (■). Results are shown from a single experiment performed in quadruplicate, which is representative of one other. ST271 and $NiCl_2$ induced greater inhibition of thrombin-induced formation of [^{3}H]inositol phosphates, relative to EGTA ($p < 0.05$); the inhibitory actions of ST271 and $NiCl_2$ were partially additive ($p < 0.05$, relative to thrombin plus ST271 and thrombin plus $NiCl_2$).

A23187 in platelets is dependent on the extracellular concentration of Ca^{2+}, because A23187-Ca^{2+} complexes are membrane impermeant (26), and therefore the effect of A23187 was investigated further in the presence of 1 mM EGTA or 1 mM Ca^{2+}. A23187 (1 μM) did not induce formation of [^{3}H]inositol phosphates under either set of conditions (Fig. 8, B and C).

Discussion

The present study was designed to investigate further the role of Ca^{2+} and occupation of the fibrinogen receptor in the regulation of phospholipase C in human platelets stimulated by G protein-coupled and tyrosine kinase-linked cell surface receptors. The results confirm earlier reports by Rittenhouse (14) and Lapetina *et al* (15) that elevation of Ca^{2+} by the ionophores A23187 or ionomycin does not induce activation of phospholipase C in the presence of inhibitors of cyclooxygenase. The mechanism of the small increase in phosphatidic acid observed by Siess and Lapetina (16) is presumably related to alternative pathways of formation of this metabolite, e.g., phospholipase D. The present results also provide evidence against the involvement of fibrinogen receptor occupancy or aggregation in the regulation of phospholipase C. The former observation contradicts that made by Sinigaglia *et al* (17) for low concentrations of thrombin, although the explanation for this discrepancy is not known.

During investigation of the role of the fibrinogen receptor in the regulation of phosphoinositide metabolism, the unexpected observation was made that EGTA inhibits activation of phospholipase C by both G protein-coupled and tyrosine kinase-linked pathways. Evidence that this effect was mediated through inhibition of Ca^{2+} entry, leading to a decrease in intracellular Ca^{2+} levels, was derived from studies with BAPTA/AM and Ni^{2+}. The much greater inhibitory effect of BAPTA, relative to EGTA, against activation of phospholipase C by both G protein-coupled and tyrosine kinase-

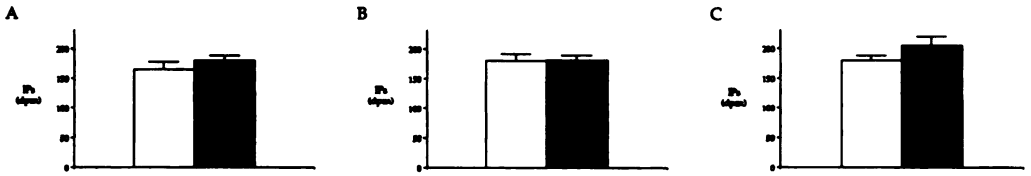

Fig. 8. Lack of Ca^{2+} ionophore A23187 stimulation of [^{3}H]inositol phosphate formation. Human platelets were labeled with [^{3}H]inositol as described in Experimental Procedures. Formation of total [^{3}H]inositol phosphates (*IPs*) was measured, after a 5-min incubation with A23187 (with stirring at 1200 rpm), by Dowex anion exchange chromatography. A23187 (2 μM)-induced formation of inositol phosphates was measured under the following conditions: no added Ca^{2+} or EGTA (A), addition of 1 mM Ca^{2+} (B), or addition of 1 mM EGTA (C). □, Basal inositol phosphate level; ■, A23187-induced inositol phosphate level. Results are shown as mean ± standard error from one experiment performed in quadruplicate, which is representative of at least two others.

linked pathways is consistent with the greater inhibition of Ca^{2+} elevation, although additional effects of BAPTA cannot be ruled out. In this context, it is noteworthy that Coorssen and Haslam (27) reported BAPTA inhibition of phospholipase D in permeabilized platelets through a pathway that did not appear to involve chelation of Ca^{2+}. The greater inhibitory effect of Ni^{2+}, relative to EGTA, may reflect an additional intracellular site of action. Ni^{2+} enters the platelet cytosol over several minutes, as judged by the slow rate of decrease in quin2 fluorescence (25), and, in line with this, a time-dependent inhibitory effect on phospholipase C activation was observed.

The mechanism through which a decrease in intracellular Ca^{2+} levels inhibits activation of phospholipase C is not known, although the inhibition of responses via both G protein-coupled and tyrosine kinase-linked pathways demonstrates that this effect is not mediated through a single isoform of phospholipase C. All isoforms of phospholipase C, however, exhibit homology in two regions, termed X and Y domains, and the regulatory effect of Ca^{2+} may reflect a dependence of either region on increased levels of this cation. Alternatively, the effect of Ca^{2+} may occur at the level of synthesis of the substrate for phospholipase C, i.e., PtdIns4,5P_2. Ca^{2+} has a number of actions on the metabolism of phospholipids in platelets, including activation of phospholipase A_2 and inhibition of formation of Ptdins4,5P_2 (15), which prevent interpretation of measurements of phospholipid levels. An additional explanation for the present observations is that Ca^{2+} binds to an inhibitory factor that ordinarily prevents access of phospholipase C isoforms to their substrate, PtdIns4,5P_2. Such an inhibitory factor was proposed originally by Rhee *et al.* (28) to explain the high activity of phospholipase C isoforms under basal conditions and the absence of an increase in activity upon stimulation.

The observation that Ca^{2+} regulates agonist-induced activation of phospholipase C provides an explanation for the inhibitory effect of ST271 on thrombin-stimulated formation of [^{3}H]inositol phosphates (11). ST271 inhibits elevation of intracellular Ca^{2+} induced by thrombin to an extent similar to that induced by EGTA, although it has a slightly greater inhibitory effect on thrombin-stimulated formation of [^{3}H]inositol phosphates. The actions of ST271 and EGTA on these responses are not additive. These data suggest that a significant proportion of the inhibitory effect of ST271 on phospholipase C activation is mediated by inhibition of Ca^{2+} entry but that ST271 must have an additional intracellular action. Ni^{2+}, which also prevents Ca^{2+} entry, similarly inhibits activation of phospholipase C to a greater extent than that induced by EGTA, suggesting that it too must have an additional intracellular action. This intracellular action increased with the time of incubation with Ni^{2+}, consistent with the slow entry of this cation into the cell, as demonstrated by the steady decrease in fluoresence of quin2-loaded platelets (25). The intracellular actions of ST271 and Ni^{2+} appear to be distinct, because their combined actions are partially additive.

The molecular basis for the intracellular action of ST271 that leads to the greater inhibition of Ca^{2+} elevation and formation of [^{3}H]inositol phosphates, relative to that induced by EGTA, is not known but may be due to inhibition of tyrosine kinases. We have reported that thrombin does not induce tyrosine phosphorylation of phospholipase C-γ isoforms in human platelets (11), although others have observed weak phosphorylation (7, 29). Alternatively, a model has been proposed in which the formation of a complex between rasGTPase-activating protein, rap1B, and phospholipase C-γ_1 at the cell membrane leads to hydrolysis of phosphoinositides (30). Formation of this complex is regulated by tyrosine phosphorylation of rasGAP and therefore would be inhibited by ST271.

In conclusion, the present study has demonstrated a role for Ca^{2+} in the regulation of phospholipase C activity by G protein- and tyrosine kinase-linked receptors, although Ca^{2+} elevation alone does not activate this pathway. These results provide an explanation for the partial inhibition of phospholipase C by the tyrphostin ST271.

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