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Human Platelet Activation Is Inhibited Upstream of the Activation of Phospholipase A_2 by U73343

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ABSTRACT. U73122 is known as an inhibitor of phospholipase C (PLC; EC 3.1.4.11). Its close structural analogue, U73343, lacks this activity and is used as a control compound. We have found that both compounds interfere with platelet signal transduction. U73122 completely abolished aggregation evoked by thrombin, TG, and collagen. Aggregation evoked by TG and collagen was also blocked by U73343, an effect due to inhibition of TxA_2 production. U73343 was a potent inhibitor of TG-evoked arachidonic acid release, but a weak inhibitor of cytosolic phospholipase A_2 ($cPLA_2$; EC 3.1.1.4) activity. Cytosolic PLA_2 activation in platelets involves protein tyrosine phosphorylation. U73343 inhibited TG- and collagen-evoked protein tyrosine phosphorylation, which can thus explain its action against these agents. These data indicate that caution is needed when using U73343 along with U73122 in the study of intracellular signalling pathways. BIOCHEM PHARMACOL 53;9:1257–1262, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. cytosolic calcium concentration; phospholipase A_2; U73122; U73343; platelet; tyrosine phosphorylation

Platelet activation and aggregation can be stimulated by the strong physiological agonists, collagen and thrombin. These ligands elicit diverse signals in platelets, including the activation of phospholipase C (PLC¶; EC 3.1.4.11). Collagen and thrombin activate the isoforms PLC_{gamma2} and, predominantly, $PLC_{\beta1}$, respectively (for review, see [1]), leading to a rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and the activation of protein kinase C (PKC¶; EC 2.7.1.37). Platelet aggregation stimulated by low levels of collagen is dependent upon the production of thromboxane A_2 (TxA_2), synthesised from a pool of arachidonic acid that is cleaved from membrane lipids by the action of cytosolic phospholipase A_2 ($cPLA_2$; EC 3.1.1.4).

The regulation of $cPLA_2$ is not fully understood. It requires Ca^{2+} and, under some circumstances, PKC may contribute to its activation. The phosphorylation of ser-505 of $cPLA_2$ by the mitogen-activated protein kinases (MAPK; EC 2.7.1.-) has also been proposed as a key regulatory event. Recent work [2, 3] has discounted the p42 and p44 MAPKs in this process, and has implicated instead the p38, stress-activated MAPK isoform [2]. However, work from one of our laboratories has shown that inhibition of p38 MAPK has only minor effects on TxA_2 synthesis and does not block the release of arachidonic acid from platelet membranes [4]. Separate lines of evidence suggest, in addition to the involvement of PKC, a role for protein tyrosine phosphorylation in the regulation of $cPLA_2$ [5, 6].

Platelets contain an abundance of intracellular tyrosine kinases (TKs; EC 2.7.1.112) [1, 7], some of which may associate with platelet receptors for collagen and for fibrinogen. However, G protein-coupled receptors such as those for thrombin or TxA_2 also elicit tyrosine phosphorylation in platelets [8, 9]. Inhibition of tyrosine phosphorylation blocks platelet aggregation. Various loci for such blockade have been identified. For example, the activation of PLC_{gamma2}, Ca^{2+} influx and the cytoskeletal rearrangements necessary for platelet shape change, secretion, and aggregation are all underpinned by increased protein tyrosine phosphorylation (reviewed in [7, 9]).

The PLC inhibitor, U73122 [10], has proved a useful tool in the study of calcium signalling mechanisms [11, 12] and other PLC-dependent processes [13]. A close structural analogue of U73122, U73343, which has negligible activity as a PLC inhibitor, is commonly used as a control compound [11–13]. One of the criteria by which the inactivity of

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¶ *Abbreviations:* $AACOCF_3$, arachidonyl trifluoromethyl ketone; aspirin, acetylsalicylic acid; $[Ca^{2+}]_i$, cytosolic calcium concentration; $cPLA_2$, cytosolic phospholipase A_2; MAPK, mitogen-activated protein kinase; PGH_2, prostaglandin H_2; PLC, phospholipase C; PMSF, phenylmethylsulfonylfluoride; PKC, protein kinase C; U73122, 1-[6-[[(17beta)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-)1H-pyrrole-2, 5-dione; U73343, 1-[6-[[(17beta)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino] hexyl]-2,5-pyrrolidinedione; TG, thapsigargin, TxA_2, thromboxane A_2.

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U73343 was initially judged was its inability to inhibit platelet aggregation [10]. However, in a study of the effects of U73122 and U73343 on platelet function we found that U73343 was a potent inhibitor of aggregation evoked by collagen and the inhibitor of the Ca^{2+} store Ca^{2+}-ATPase, TG. We have investigated this inhibition and present evidence that it occurs upstream of the activation of $cPLA_2$, at a step that may involve tyrosine phosphorylation.

MATERIALS AND METHODS

Human platelet suspensions at a density of 1.0×10^8/mL in HEPES-buffered saline (pH 7.45; supplemented with 0.1% w/v bovine serum albumin) were prepared as previously described [14]. Where required, aspirinated platelets were prepared by addition of 200 μM aspirin to citrated platelet-rich plasma prior to centrifugation.

Platelet aggregation was monitored in a Chronolog (Havertown, PA., USA) aggregometer under stirring at 900 rpm at 37°C [15].

TxA_2 production was quantified as its product TxB_2 in platelet-free supernatants, centrifuged from platelet suspensions activated under stirring, using a kit from Du Pont (Boston, MA, USA) as previously described [15].

Protein tyrosine phosphorylation was detected by gel electrophoresis and Western blotting with a specific anti-phosphotyrosine antibody as previously described [14].

To study release of [^{14}C]arachidonic acid, platelet-rich plasma from two donors (5.6×10^9 platelets) was incubated with a sonicated dispersion of sodium [1-^{14}C]arachidonate (35 kBq each) in the presence of 1 μM prostaglandin E_1 under gentle shaking at 37°C for 2 hr, essentially as described for rat platelets [16]. The radiolabelled platelets were collected by centrifugation, washed, and finally resuspended in a HEPES-buffered saline as above. Aliquots of 2 mL were activated with the desired agonist under stirring at 900 rpm at 37°C. Reactions were stopped by addition of 5 mM EDTA and the cells pelleted in an Eppendorf microcentrifuge at 16000 × *g* for 90 sec. The radioactivity released into the supernatants, which contained exclusively free arachidonate and its oxygenated products, was counted. Lipids were extracted from the platelet pellets with acidic chloroform and methanol. Finally, free radioactive arachidonic acid was separated from phosphatidylcholine by thin-layer chromotography as previously described [16]. Silica gel plates were eluted with the upper (organic) phase of an ethylacetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100 v/v) mixture [17]. In both stimulated and unstimulated platelet pellets, >98% of the radioactivity colocalised with the phospholipids and only 1% with the triacylglycerols. Thus, the platelet pellets were almost devoid of free arachidonate and its oxygenated products.

For determination of PLA_2 activity, washed platelets (2×10^8/sample) were incubated with 0.5 mM $CaCl_2$ for 2 min under slow stirring or activated under these conditions with the desired agonists before dilution (1:1) with a buffer containing 10 mM HEPES, 1.5 mM EDTA, 100 mM KCl, 0.5 mM PMSF, and 4.2 μM leupeptin, pH 7.45. The diluted suspensions were then sonicated on ice for 6 min (30 sec on, 30 sec off). Vesicles were prepared from 1-stearoyl-2-[^{14}C]arachidonyl-phosphatidylcholine in 50 mM Tris/HCl (pH 8.1). Samples from the platelet sonicates (200 μL) were incubated with 1 vol of phospholipid vesicles and 6 vol of 50 mM Tris/HCl buffer (pH 8.1) for 30 min at 37°C. Reactions were started by addition of 2.5 mM $CaCl_2$ and stopped by the addition of chloroform/methanol. Inhibitors were added as required. Lipids were extracted according to [18] in the presence of 1 M HCl. Finally, radioactive phospholipids were separated from free arachidonic acid by thin-layer chromatography. Each assay contained 825 Bq.

U73122, U73343, and $AACOCF_3$ were from Biomol (Plymouth Meeting, PA., USA). [1-^{14}C]arachidonic acid and 1-stearoyl-2-[1-^{14}C]arachidonyl-phosphatidylcholine (each 2.0 GBq/mmol) was from Amersham (Amersham, UK) and unlabelled sodium arachidonate from Sigma (St. Louis, MO, USA). Collagen (type 1 fibres) was from Hormon-Chemie (Munich, Germany).

RESULTS AND DISCUSSION

The effects of U73122 and U73343 on platelet aggregation are shown in Fig. 1. The PLC inhibitor, U73122 (5 μM), completely inhibited aggregation evoked by thrombin (10 nM) (Fig. 1A). This was as expected, because this concentration of U73122 abolishes the thrombin-evoked rise in $[Ca^{2+}]_i$ [19], and the thrombin-evoked response is believed to depend on the activation of one or more PLC isoforms [1]. Thrombin-evoked shape change was unaffected by U73122 (5 μM). Agonist-evoked shape change in the absence of a detectable rise in $[Ca^{2+}]_i$ and, therefore, presumably without significant PLC activation has been reported before [20, 21]. The mechanism of this Ca^{2+}-independent shape change appears also to be independent of activation of PKC [22] and remains unknown. The putatively inactive analogue of U73122, U73343 (20 μM), was, as expected, without effect on thrombin-evoked aggregation (Fig. 1A).

Aggregation evoked by the Ca^{2+}-ATPase inhibitor, thapsigargin, was also abolished by U73122 (Fig. 1B). This finding is compatible with earlier evidence that full release of stored Ca^{2+} in platelets by thapsigargin requires a calcium-dependent activation of PLA_2 and the generation of TxA_2 [23]. Thus, the full response is dependent on the activation of PLC-β_1 via the TxA_2/PGH_2 receptor [1] in order to generate inositol 1,4,5-trisphosphate, which appears to release Ca^{2+} from a TG-insensitive store [1, 23]. Although aggregation can be stimulated by an ionophore-mediated rise in $[Ca^{2+}]_i$ alone [24], the threshold $[Ca^{2+}]_i$ required is substantially higher than when PKC is also stimulated by DAG production [25]. A reduced elevation in $[Ca^{2+}]_i$ and reduced production of DAG in response to

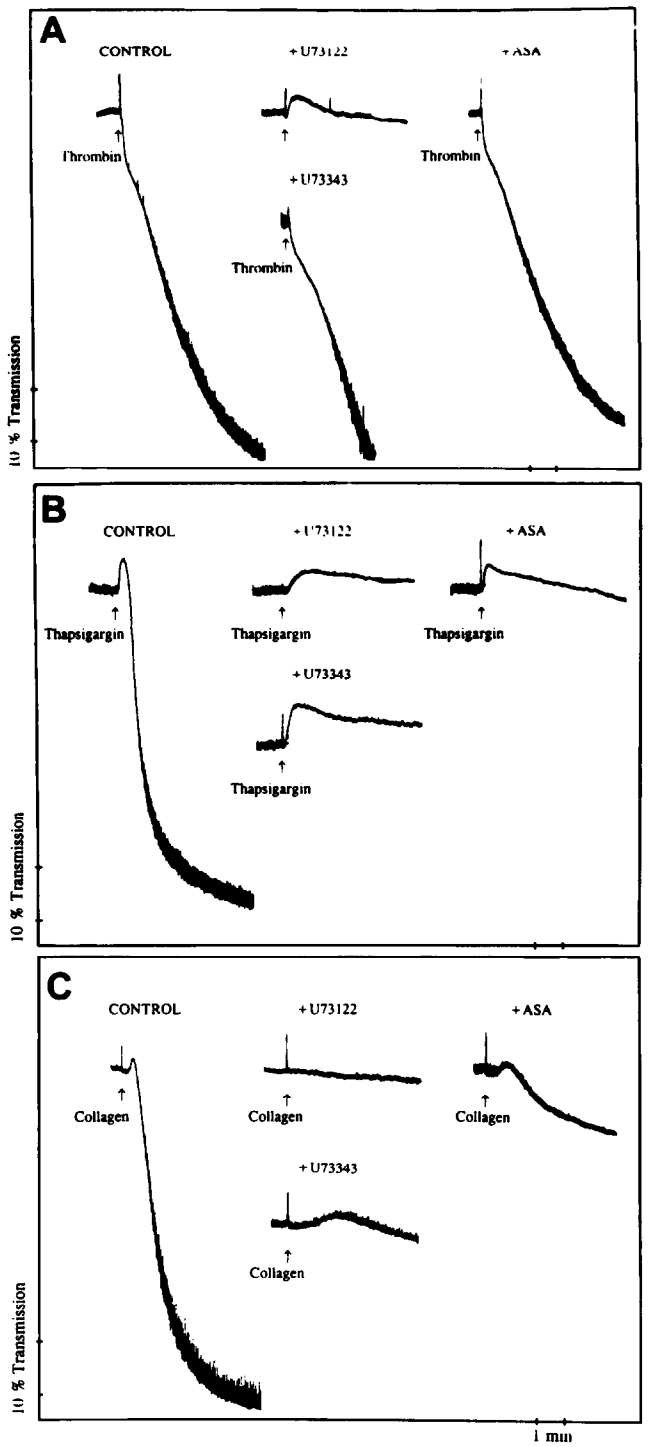

FIG. 1. Effects of U73122, U73343, and aspirin on platelet aggregation. Traces show optical density of stirred platelet suspensions stimulated by the addition of (A) thrombin (10 nM), (B) TG (100 nM), or (C) collagen (5 μg/mL) under control conditions, or after treatment with U73122, U73343, or aspirin as indicated above traces. U73122 (5 μM) or U73343 (20 μM) were added 2 min prior to the agonist. Aspirinated platelets were prepared by addition of 200 μM acetylsalicylic acid to aliquots of platelet-rich plasma before centrifugation and platelet collection. Records typical of at least four experiments.

the secondary generation of TxA_2 could thus explain the inhibitory effect of U73122 on thapsigargin-evoked aggregation. The dependency of thapsigargin-evoked aggregation on TxA_2 generation is illustrated by the fact that blockade of cycloxygenase using aspirin (100 μM) also inhibits the response (Fig. 1B). U73343 (20 μM) was also found to inhibit TG-evoked aggregation (Fig. 1B). This was unexpected, because U73343 is reported to have little effect on PLC [11]. U73122 (5 μM) abolished aggregation evoked by collagen (5 μg/mL) (Fig. 1C). As with TG, this effect could be attributed to blockade of PLC-β_1 activation by TxA_2 because collagen-evoked aggregation was also substantially reduced by aspirin (100 μM) (Fig. 1C). Unexpectedly, aggregation evoked by collagen (5 μg/mL) was also abolished by U73343 (20 μM) (Fig. 1C).

The finding that U73343 inhibited aggregation evoked by TG and collagen was investigated further. Because this inhibition was similar to that obtained when the generation or action of TxA_2 was blocked using aspirin or U73122, we determined the effect of all three agents on TxB_2 formation (Table 1). U73343 (20 μM), U73122 (5 μM), and aspirin (100 μM) all substantially reduced TxB_2 production evoked by thrombin (10 nM), TG (100 nM), and collagen (5 μg/mL). Thus, it appears that the inhibitory effect of U73343 on aggregation evoked by TG and collagen can be explained by inhibition of TxA_2 production. In agreement with this, the TxA_2 analogue, U46619 (100 nM) was found to fully restore aggregation when added after TG (100 nM) in the presence of U73343 (20 μM) (data not shown). TxA_2 production evoked by thrombin was also inhibited by U73122 and U73343, but thrombin-evoked aggregation is independent of this secondary pathway, as demonstrated by its insensitivity to aspirin (Fig. 1A). Furthermore, it appears that the inhibitory effects of U73122 on TG- and collagen-evoked responses could be due more to inhibition of the production of TxA_2 than its action on $[Ca^{2+}]_i$ (Table 1).

To investigate whether the inhibitory effect of U73343 on agonist-evoked TxA_2 production was due to inhibition of PLA_2 activation, the release of [^{14}C]arachidonic acid

TABLE 1. Effects of U73122, U73343, and aspirin on thromboxane A_2 formation

Agonist	Inhibitor	Formation of TxB_2 (% of control ± SE)
Thrombin (10 nM)	U73122 (5 μM)	2.6 ± 1.8
	U73343 (20 μM)	31.8 ± 10.1
	Aspirin	0.5 ± 0.5
TG (100 nM)	U73122 (5 μM)	3.2 ± 0.3
	U73343 (20 μM)	9.5 ± 6.2
	Aspirin	0 ± 0
Collagen (5 μg/ml)	U73122 (5 μM)	2.6 ± 1.0
	U73343 (20 μM)	2.6 ± 1.0
	Aspirin	0 ± 0

TxB_2 was assayed in supernatants from centrifuged samples taken from aggregation experiments as in Fig. 1. Reactions were terminated 5 min after addition of agonist by addition of 5 mM EDTA. U73122, U73343, and aspirin pretreatments as for Fig. 1. Results shown are means ± SE of three to four experiments.

evoked by thrombin and TG was determined (Fig. 2). U73343 (20 μM) substantially reduced the [^{14}C]arachidonic acid release evoked by both agents. Aspirin had little effect on the [^{14}C]arachidonic acid release evoked by either agent (Fig. 2), indicating that the action of U73343 was not secondary to inhibition of cycloxygenase.

The action of U73343 on [^{14}C]arachidonic acid release from platelet phospholipids cannot be attributed to an action on PLA_2 activity alone. In platelet sonicates prepared from cells stimulated with thrombin (10 nM) or TG (100 nM), U73343 (20 μM) only reduced the release of [^{14}C]arachidonic acid from 1-stearoyl-2-[^{14}C]arachidoyl-phosphatidylcholine vesicles to 70.7 or 76.9% of vehicle treated controls, respectively (means of duplicate determinations). The specific inhibitor of cytosolic PLA_2, $AACOCF_3$ (10 μM) [26], reduced [^{14}C]arachidonic acid release by sonicates to 2.9 ± 1.8% of controls (mean ± SEM of three determinations in triplicate). Thus, the PLA_2 activity in platelet sonicates is almost entirely attributable to the cytosolic isoform of the enzyme.

It has recently been shown that $cPLA_2$ activation may involve protein tyrosine phosphorylation, either directly or downstream from kinases themselves regulated by tyrosine phosphorylation [2, 3, 5, 6]. Thus, finally we investigated the effects of U73343 on this process. As shown in Fig. 3, platelet activation by TG, collagen, and thrombin all resulted in substantial protein tyrosine phosphorylation. The actions of TG or collagen were inhibited by U73343 (20 μM), while that evoked by thrombin was less affected. Aspirin caused a partial inhibition of tyrosine phosphorylation stimulated by either thapsigargin or collagen (data not shown), indicating a role for TxA_2 in amplifying tyrosine kinase activity in platelets. However, in the presence or absence of aspirin, we observed substantial inhibition by U73343 of tyrosine phosphorylation stimulated by these ligands. The inhibition by U73343 of protein tyrosine phosphorylation stimulated by either TG or collagen was not dependent on platelet aggregation, because these measurements were made using aspirin-treated platelets. Aspirin abolished aggregation stimulated by TG (Fig. 1B), and that stimulated by collagen was substantially reduced (Fig. 1C), so that the inhibition of tyrosine phosphorylation shown in Fig. 3 was observed at times well in advance of the onset of such aggregation as remained. These results indicate that the inhibitory effects of U73343 on the activation of $cPLA_2$, thromboxane A_2 production, and thus aggregation evoked by TG and collagen in human platelets could be the result of inhibition of the protein tyrosine phosphorylation evoked by these agents. Such an effect might also explain the inhibition of the thrombin-evoked activation of $cPLA_2$ and TxA_2 production by U73343. It is quite striking that while U73343 had a modest inhibitory effect on the tyrosine phosphorylation of 70 and 130 kDa proteins evoked by thrombin, it substantially reduced the intensity of tyrosine phosphorylation of a protein band of approximately 60 kDa evoked by each of the agonists investigated here. This substrate may be one of the *src* family of tyrosine kinases, and its potential role in the regulation of $cPLA_2$ is currently under investigation. We have also reported that U73122 affects platelet protein tyrosine phosphorylation, increasing tyrosine phosphorylation itself, but blocking subsequent agonist-evoked changes [19].

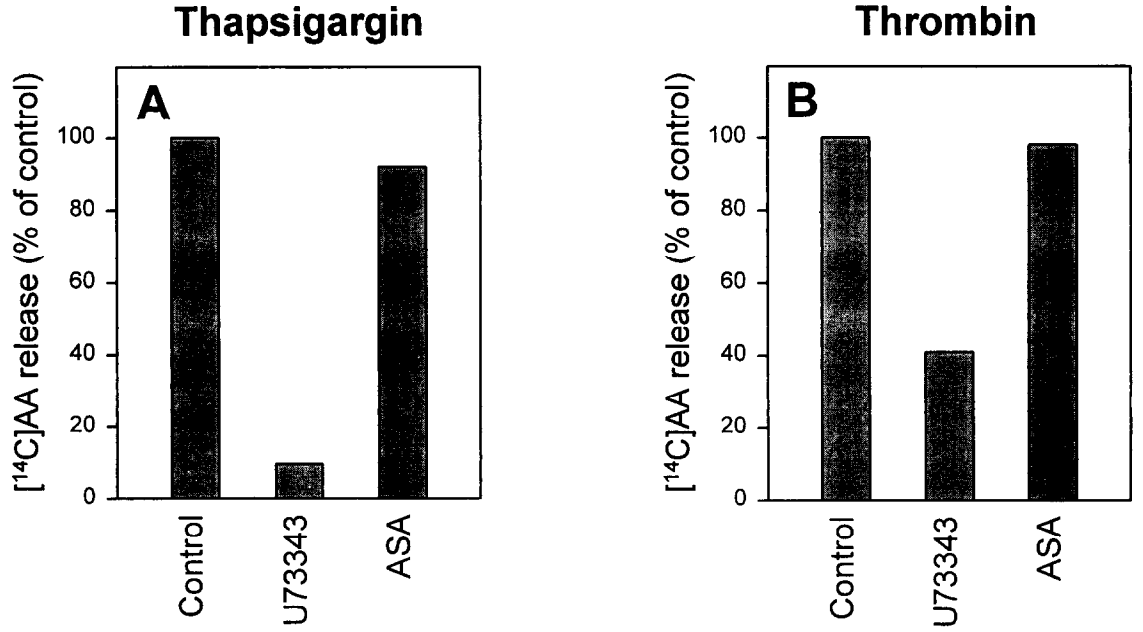

FIG. 2. Effects of U73343 and aspirin on arachidonic acid release. Release of [^{14}C]arachidonate (determined as under Methods) evoked by (A) TG (100 nM) or (B) thrombin (10 nM) after treatment with U73343 (20 μM) or aspirin is shown as the percentage of release under control conditions. Treatments with U73343 or aspirin as for Fig. 1. Results are means of two independent experiments in triplicate.

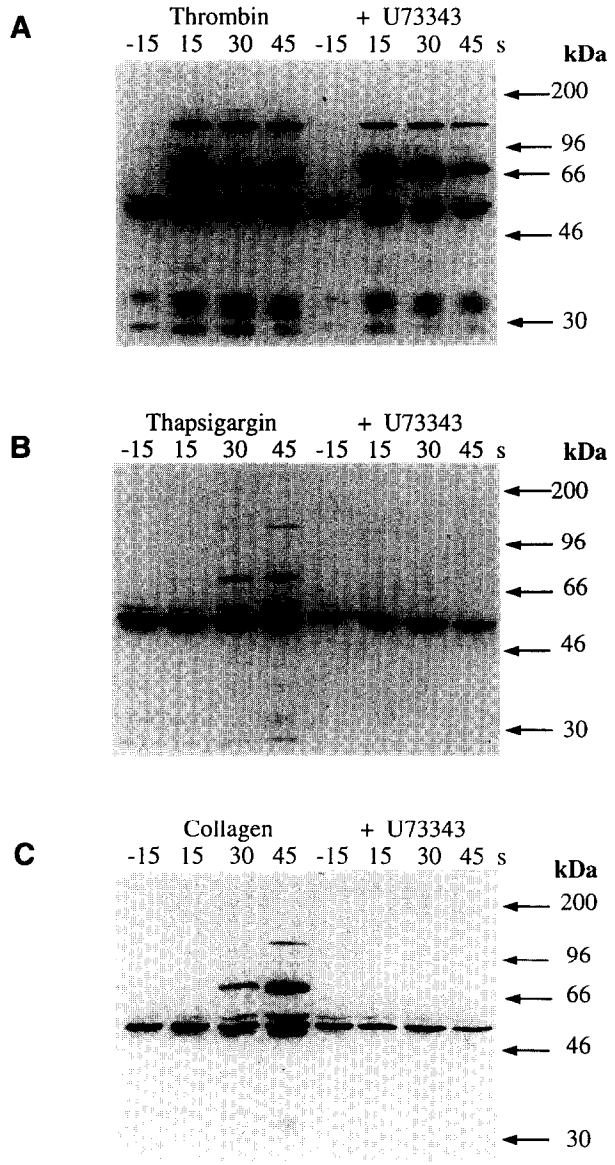

FIG. 3. Effects of U73343 on protein tyrosine phosphorylation. Aspirinated platelets were stimulated in a stirred cuvette by (A) thrombin (10 nM), (B) TG (100 nM), or (C) collagen (5 μg/mL). Platelets were pretreated with U73343 (20 μM) as indicated. Samples were withdrawn at the times indicated, stopped with Laemmli's buffer, and Western blots were prepared as described in Materials and Methods. These experiments were repeated on at least three different platelet preparations.

It is concluded that U73122 has effects on platelet activation that can be attributed to its established inhibitory effect on PLC [10], but additional actions may be ascribed to inhibition of TxA_2 formation. U73343, although reported to have little effect on PLC, also has effects on platelet activation that can be attributed at least in part to inhibition of tyrosine kinase activity. The effects reported here indicate that caution is needed when using U73343 as an inactive control for U73122 in the investigation of signalling pathways.

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