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SHORT COMMUNICATION

Evidence for Separate Effects of U73122 on Phospholipase C and Calcium Channels in Human Platelets

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ABSTRACT. U73122 ({1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)exyl]-1H-pyrrole-2,5-dione}) is generally used as a selective inhibitor of phospholipase C (PLC) and the related rise in cytosolic Ca^{2+}. Recently, by using hepatocytes, it was suggested that its action sites are different for PLC activation and increase in Ca^{2+} concentration. To verify whether U73122 has different sites for inhibiting PLC activation and calcium responses in human platelets, aggregation, Mn^{2+} influx, cytosolic Ca^{2+} increase and PLC activation were studied in response to thrombin and the synthetic agonist of the thromboxane receptor U46619 (9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$). With both agonists, U73122 inhibited aggregation, Mn^{2+} influx and the enhancement of cytosolic calcium at concentrations of 2 μM or lower, while 10 μM was necessary to inhibit PLC activation. Our results suggested that U73122 is much more active in antagonizing Ca^{2+} channels, both the intracellular ones, which are activated by formation of inositol 1,4,5 P_3 and those present on plasma membrane, than in reducing the activation of PLC. BIOCHEM PHARMACOL **56**;11:1481–1484, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. platelets; phospholipase C; calcium mobilization; calcium channels

The cell permeable aminosteroid U73122§ is generally used as an inhibitor of PLC [1–3]. In fact, it was previously demonstrated that U73122 reduces IP3 formation as well as PLC-dependent processes in neutrophils [4]. However, using the same cell type, it was later shown that U73122 not only dose-dependently inhibits the release of calcium from intracellular stores but also blocks the influx of calcium across the plasma membrane; moreover, in electropermeabilized cells U73122 did not impair either the tyrosine phosphorylation of PLCγ_2 or IP3 formation, whereas it was able to inhibit the IP3-mediated release of calcium [5]. Studies with other cell types have also led to the suggestion that U73122 inhibits Ca^{2+} channels by a mechanism different from that inhibiting PLC, probably by an action on a protein involved in the regulation of a Ca^{2+} inflow channel or of store-operated Ca^{2+} inflow [6–8].

As regards human blood platelets, it was previously proposed that U73122 acts as a selective inhibitor of PLC [9]; recently, based on experiments measuring the rises in cytosolic calcium concentration ($[Ca^{2+}]i$) evoked by thrombin, collagen and thapsigargin, Heemskerk and coworkers suggested that U73122 inhibits the activity of both the β and γ isoforms of PLC [10]. However, these authors also suggested that U73122, as well as its close analogue U73343, is not a selective inhibitor of PLC, as it has a direct effect on tyrosine phosphorylation of platelet proteins [10].

The aim of the present study was to evaluate whether the inhibition of Ca^{2+} influx and PLC activation by U73122 is the consequence of a single activity or if U73122 has different sites of action on the signal transduction pathways in human platelets. Aggregation, manganese influx, Ca^{2+} mobilization and Ins (1,3,4) P_3 production in response to thrombin and to the synthetic agonist of the thromboxane A2 receptor U46619 were studied. The results support the idea that U73122 inhibits calcium channels and PLC activation by different mechanisms in human platelets, and that the inhibitory effect on platelet aggregation is not the consequence of PLC suppression.

MATERIALS AND METHODS

Platelet Preparation

Platelet rich plasma was obtained after centrifugation (180 *g* for 15 min) of blood samples taken by venipuncture from healthy volunteers into acid/citrate/dextrose (ACD) [11].

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§ *Abbreviations:* GFP, gel-filtered platelets; IP3, inositol 1,4,5 P_3; PLC, phospholipase C; U46619, 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$; and U73122, {1-[6-((17β-3-methoxyestra-1,3,5(10-)trien-17-yl)amino)exyl]-1H-pyrrole-2,5-dione}.

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Platelet-rich plasma was recentrifuged at 800 *g* for 20 min and the pellet was resuspended in 0.5 vol of autologous platelet poor plasma. The concentrated platelets were incubated with aspirin (1 mM) (Sigma) for at least 15 min at 37° and with either the fluorescent indicator Fura-2-AM (4 μM) (Molecular Probes) for 30 min at 37° and then for 15 min at room temperature, or with [^{32}P] (1 mCi/1 × 10^9 cells) (Amersham) for 1 hr at 37°. Platelets were washed by gel filtration (GFP) on Sepharose 2B (Pharmacia) using Ca^{2+}-free Tyrode's buffer containing 0.2% BSA (fraction V Sigma), 0.1% glucose and 10 mM HEPES (pH 7.35).

Platelet Aggregation

In vitro platelet aggregation was evaluated according to Born [12] in a four sample Aggrecorder II (Menarini) at 37° using siliconized glass cuvettes under continuous stirring at 1000 rpm. Fibrinogen (1 mg/mL) was added to GFP (2 × 10^8 cells/mL) before the synthetic agonist of the endoperoxide-thromboxane receptor U46619 (1 μM) (Cayman Chemicals) and human thrombin (1 U/mL) (Sigma).

Changes in Intraplatelet Calcium Concentration

An aliquot (0.5 mL) of Fura 2-loaded GFP (2 × 10^8 cells/mL) was added to cuvettes thermostatically maintained at 37° and continuously stirred. The fluorescence changes were monitored with a SFM 25 fluorimeter (Kontron), set at 340 nm excitation and 510 nm emission. Differences in free calcium concentrations (Δ) between the unstimulated and stimulated GFP were calculated using as F_{min} the value determined after the addition of digitonin (50 μM) (Sigma) in the presence of EGTA (2 mM) and Tris base (20 mM), as F_{max} the value measured after the addition of excess $CaCl_2$ (10 mM), and a K_d of 224 nM after correction for extracellular dye [13]. The base level was never higher than 100 nM.

In order to evaluate platelet calcium influx, we exploited the capability of Mn^{2+} to use calcium channels to enter into platelets and quench Fura-2 fluorescence. In the presence of $CaCl_2$ (1 mM), $MnCl_2$ (50 μM) was added to the cell suspension before the agonist. The fluorescence changes were monitored at 360 nm excitation to eliminate the enhancement of fluorescence due to calcium mobilization, as well as at 510 nm emission. The results were expressed as the percentage of reduction of Fura-2 fluorescence induced by the agonists compared to the capability of Mn^{2+} to totally quench the Fura-2 fluorescence after the addition of digitonin (50 μM) [14].

Phospholipase C Activation

The stable metabolite of phosphatidyl inositol 4,5 bisphosphate, Ins 1,3,4 P_3, was used as indicator of phospholipase C activation. Its production was analyzed 30 sec after the addition of the agonists in [^{32}P]-labeled, aspirinated platelets (1 × 10^9 cells/mL) resuspended in Ca^{2+} and phosphate-free Tyrode's buffer. The activation was stopped with perchloric acid (0.44 N). The Ins (1,3,4) P_3 formation was evaluated as previously described [15]. The results are reported as the difference between cpm at time 0 and 30 sec.

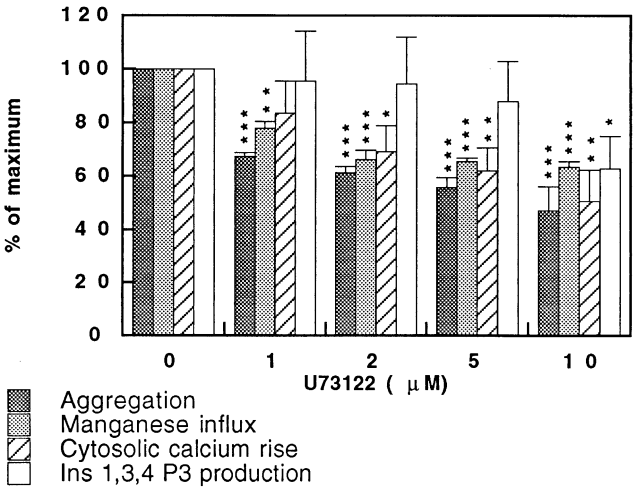

FIG. 1. Effect of scalar doses of U73122 (1–10 μM for 3 min at 37°) on thrombin (1 U/mL)-induced platelet aggregation, Mn^{2+} influx, cytosolic calcium increase and Ins (1,3,4) P3 production. The results are expressed as the means ± SD (four experiments performed) of the % of maximum obtained. The maxima were: 90.5 ± 2.9% for platelet aggregation; 25.8 ± 4.1% for Mn^{2+}-induced Fura-2 fluorescence reduction; 748 ± 255 Δ nM for cytosolic Ca^{2+} concentration; and 6328 ± 3277 Δ cpm Ins 1,3,4 P_3 production. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; no sign = not significant.

For all the above-described experiments, U73122 (1 mM in DMSO + saline 1/1 v/v; Calbiochem-Novabiochem Corp.), or its vehicle, was preincubated with platelets for 3 min at 37° before the stimulus.

RESULTS

Figures 1 and 2 show that U73122 (1–10 μM) dose dependently inhibited the maximum platelet aggregation, Mn^{2+} influx, changes in intracellular calcium concentration and PLC activation induced by 1 U/mL of thrombin (Fig. 1) and 1 μM U46619 (Fig. 2). Platelet aggregation and manganese influx induced by both agonists were the only responses significantly reduced after preincubation with 1 μM U73122. Increases in intracellular calcium in response to thrombin or U46619 showed a significant reduction only after addition of 2 μM U73122. Similar results were obtained when platelets were treated with EGTA to investigate the calcium mobilization from intracellular stores. In fact, U73122 (1, 2 and 5 μM) reduced, in a dose-dependent manner, the Δ calcium mobilization in response to both thrombin (551 ± 44 ΔnM, 517 ± 39 ΔnM and 464 ± 48 ΔnM respectively, versus 748 ± 59 ΔnM of untreated platelets) and U46619 (344 ± 37 ΔnM, 308 ± 25 ΔnM and 230 ± 39 ΔnM respectively, versus 410 ± 28 ΔnM of untreated platelets). As regards PLC inhibition,

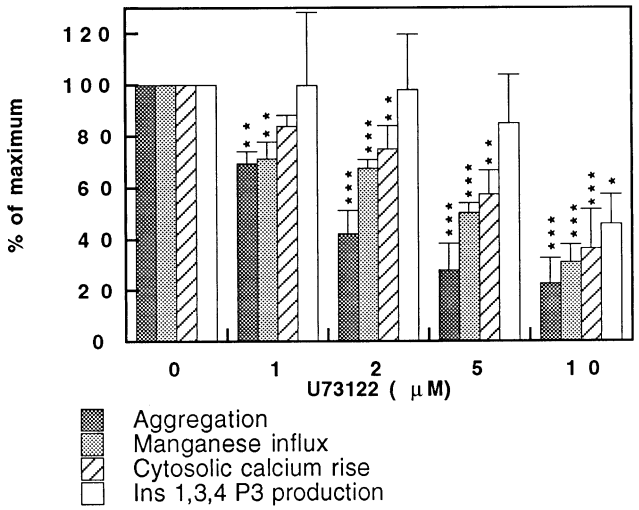

FIG. 2. Effect of scalar doses of U73122 (1–10 μM for 3 min at 37°) on U46619 (1 μM)-induced platelet aggregation, Mn^{2+} influx, cytosolic calcium increase and Ins (1,3,4) P3 production. The results are expressed as the means ± SD (four experiments performed) of the % of maximum obtained. The maxima were: 86.1 ± 6.4% for platelet aggregation; 14.5 ± 3.2% for Mn^{2+}-induced Fura-2 fluorescence reduction; 430 ± 58 Δ nM for cytosolic Ca^{2+} concentration; and 958 ± 378 Δ cpm for Ins 1,3,4 P_3 production. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; no sign = not significant.

the dose-response curve to U73122 was shifted to the right, with a significant reduction observed only at 10 μM with both thrombin and U46619.

DISCUSSION

Our results show that U73122 is more active in antagonizing Ca^{2+} channels, both the intracellular ones which are activated by IP_3 and those present on plasma membrane, than in inhibiting PLC. In fact, this drug significantly reduced platelet aggregation, the influx of Mn^{2+}, and Ca^{2+} mobilization at a concentration of 1 to 2 μM, while the production of the stable metabolite of phosphatidylinositol 4,5 P_2, Ins 1,3,4 P_3, was significantly reduced only by using U73122 at the concentration of 10 μM.

Other authors have previously suggested that U73122 is a selective inhibitor of PLC in platelets and that the inhibitory effects on both calcium mobilization and aggregation may depend on the inhibition of PLC [9]. These authors also observed a discrepancy between the doses active on PLC and those inhibiting Ca^{2+} movements; however, their results might have been due to the difference in the concentration of thrombin used to assess the effects of U73122 on agonist-induced PLC activation (2 U/mL) and that (0.2 U/mL) used to evaluate the inhibitory action of this agent on cytosolic Ca^{2+} increases. In our experiments, the same concentration of thrombin (1 U/mL) was used to assess the effects of U73122 on all the parameters studied.

Our conclusions are in agreement with the observations by Hellberg *et al.* [5] and by Berven and Barritt [7] with different cell types, neutrophils and hepatocytes, suggesting that the action sites of U73122 were different for Ca^{2+} channels and PLC. Besides its effects on intraplatelet Ca^{2+} increases, the inhibitory effect of U73122 on platelet aggregation may depend on the ability of this compound to interfere with tyrosine phosphorylation of platelet proteins [9], a biochemical pathway important in determining fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ [16].

We may conclude then that: 1) U73122 has different inhibitory sites for Ca^{2+} channels and PLC in human blood platelets, additional evidence that it cannot be considered as a selective inhibitor of PLC; and 2) both plasma membrane and dense tubular system Ca^{2+} channels are antagonized by U73122.

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