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EFFECT OF GENISTEIN, A TYROSINE KINASE INHIBITOR, ON U46619-INDUCED PHOSPHOINOSITIDE PHOSPHORYLATION IN HUMAN PLATELETS

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Summary. Recent evidence suggests that the agonist-induced formation of phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) via PI and PIP kinases may play an important role in transmembrane signalling. In the present work, the effect of genistein, a specific inhibitor of protein-tyrosine kinase, on phosphoinositide phosophorylation was studied in human platelets stimulated with the endoperoxide analogue, U46619. At 100μM concentration, genistein, but not the related compounds, flavone and biochanin A, which possess only weak anti-protein-tyrosine kinase activity, significantly inhibited the U46619-induced accumulation of [^{3}H]PIP (by 71%) and [^{3}H]PIP_2. These data suggest that phosphoinositide phosphorylation may be regulated, in part, by tyrosine phosphorylation in U46619-stimulated platelets.

A great deal of attention has been focused on the rapid receptor-mediated hydrolysis of phosphatidylinositol 4,5 - bisphosphate (PIP_2) by phospholipase C in many cell types including platelets. The products of this hydrolysis, inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DG), serve as second messenger molecules by mobilizing intracellular Ca^{+2} (1) and activating protein kinase C (2), respectively. Recent evidence suggests that the agonist-induced formation of phosphatidylinositol 4-phosphate (PIP), and PIP_2 via PI and PIP kinases may also play an important role in cellular signal tranduction. In addition to providing substrate for second messenger generation (3-5), the increased synthesis of PIP and

Abbreviations used are: ANS, 8-anilino-1-napthalene-sulfonic acid; DMSO, dimethyl sulphoxide; dpm, disintegrations per minute; DG, diacylglycerol; EGF, epidermal growth factor; IP3, inositol 1,4,5 trisphosphate; PI, phosphatidylinositol; PIP, phosphatidyinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; SE, standard error.

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PIP_2 following agonist-stimulation (4,6,7) may be of significance in regard to the regulation of Ca^{+2} homeostasis (8,9), and actin polymerization (10) through interaction with profilin (11) and gelsolin (12).

Relatively little is known about the regulation of PI and PIP kinase activities in agonist-stimulated cells. In platelets, phorbol esters strongly stimulate PIP synthesis (13,14), thereby suggesting the involvement of protein kinase C in this response. More recently, protein-tyrosine kinases involved in cell proliferation and transformation have been associated with PI kinase activity in various growth factor (15,16), and oncogenically transformed (15,17,18) cells. Interestingly, platelets have also been shown to possess considerable pp60 $^{c\text{-}src}$-related protein-tyrosine kinase activity (19-22), which is stimulated by thrombin (21-23), and collagen (23). Given the recent availability of genistein, a specific tyrosine kinase inhibitor (24), and its use in cultured cells to determine protein-tyrosine kinase mediated events (25), we have evaluated the potential for this compound to influence agonist-induced accumulation of PIP and PIP_2 in platelets stimulated with the endoperoxide analogue, U46619.

Materials and Methods

Materials. The following materials and sources were used: genistein (Biomol, Plymouth Meeting, PA), flavone, PIP and PIP_2 standards, 8-anilino-1-naphthalene-sulfonic acid (ANS) (Sigma, St. Louis, MO), biochanin A (Aldrich Chemical Co., Milwaukee, WI), endoperoxide analogue (U46619) (Upjohn Co., Kalamazoo, MI), [^{3}H]inositol (18.2 Ci/mMol) (Amersham Canada Ltd., Oakville, ON).

Platelet isolation and prelabelling. Blood samples were collected from an antecubital vein of healthy subjects who had not taken any medication in the previous two weeks. Washed platelet suspensions were prepared according to the method of Mustard et al.(26), with the following exception: wash buffer 1 (in which platelets were prelabelled with 25µCi of [^{3}H]inositol for 2hr at 37°C) contained 4mM $MnCl_2$ and 0.55 mM dextrose in place of 2 mM $MgCl_2$ and 5.55 mM dextrose, respectively. Following the final resuspension, platelet concentrations were adjusted to give a final concentration of 5 x 10^8 platelets/ml (after additions).

Platelet incubations. One ml aliquots of platelet suspension (final volume after additions) were stirred in a Payton (Buffalo, NY) aggregometer for 1 min at 900 rpm and 37°C. Genistein, flavone, biochanin A, or vehicle (DMSO) (5µl) were added 2 min prior to the addition of 1µM U46619 (in 10µl of saline:ethanol 137:5, v/v). Reactions were terminated after 3 min by the addition of 3.75 ml chloroform: methanol: concentrated HCl at 20:40:1 (v/v/v) and the phospholipids extracted.

Extraction and analysis of phosphoinositides. Phospholipids including PIP and PIP_2 were extracted according to the method of Allan and Mitchell (27). Unlabelled PIP and PIP_2 standards were added to the lipid extracts and the phosphoinositides were separated by thin layer chromatography (28). Lipids were visualized under ultraviolet light after spraying the plates with ANS. Individual bands were scraped into scintillation vials and counted in a Beckman 7800 liquid scintillation counter.

Statistical analysis. The data were log-transformed and analyzed using analysis of variance. Specific differences for pre-planned comparisons were determined using a paired student t-test (29).

Results and Discussion

As shown in Table 1, U46619 (1μM) stimulation of human platelets significantly increased both [^{3}H]PIP (by 77.7%) and [^{3}H]PIP_2 (by 26.5%), and decreased the labelling in phosphatidylinositol (PI) (by 7.7%). Two min preincubation with 100 μM genistein, as used previously to inhibit protein-tyrosine kinase in cultured cells (25), inhibited all of these agonist-induced responses. In the case of [^{3}H]PIP, the agonist-induced accumulation was inhibited by 71% in the presence of genistein.

Table 1. Effect of genistein and related compounds on [^{3}H]inositol labelled phosphoinositides following U46619 stimulation[1]

Incubation Conditions	Phosphoinositide		
	PI	PIP	PIP2
U46619 (1μM) alone[a]	92.3±2.6	177.7±19.5	126.5±9.0
U46619+Genistein(100μM)	99.6±2.2*	122.2±8.9**	79.1±0.6***
U46619+Flavone(100μM)	92.5±0.5	194.3±27.1	135.7±5.9
U46619+Biochanin A(100μM)	92.3±1.7	202.0±22.0	135.3±3.4

[1]Results are expressed as a percentage ± SE. of dpm in PI, PIP, and PIP_2 of corresponding unstimulated (minus U46619) platelets. The average dpm in PI, PIP, and PIP_2 of resting (unstimulated) platelets was 19261, 1328, and 1170, respectively. n=4 for genistein; n=3 for flavone and biochanin A.
[a]The radioactivities in the PI, PIP, and PIP_2 were different ($p<0.05$) from the corresponding unstimulated platelets.
*,**,***Significantly different from U46619 alone; *($p<0.05$), **($p<0.005$), ***($p<0.0005$).

Two related compounds, flavone and biochanin A, which possess only weak anti-tyrosine kinase activity (24), did not significantly affect any of the U46619-induced changes in phosphoinositide labelling (Table 1). Genistein, also at 100μM concentration, inhibited U46619-induced platelet aggregation in all subjects tested, without a notable effect on the shape change response (not shown).

The relatively high levels of protein-tyrosine kinase activity in platelets, and its stimulation by thrombin, has led to the suggestion that protein phosphorylation on tyrosine may play a role in platelet signal transduction (23). We report herein upon a biochemical response (phosphoinositide phosphorylation) which appears to be mediated via a protein-tyrosine kinase in agonist-stimulated platelets.

The phosphoinositide kinase associated with protein-tyrosine kinases in growth factor (15), and insulin (16) stimulated cells is believed to be the so-called Type I PI kinase (17), which phosphorylates phosphoinositides (30) on the 3 position of the inositol ring. Walker et al. (31), on the other hand, have described a PI 4-kinase which they believe to be identical to the PI kinase activity responsible for increased levels of PIP in epidermal growth factor (EGF)-stimulated A431 cells (4). Although no evidence was provided as to whether the PI kinase described by Pike and colleagues may be regulated by tyrosine phosphorylation, the EGF receptor, as well as other growth factor receptors, possess intrinsic protein-tyrosine kinase activity (32). Very recently, Nolan and Lapetina (7) identified the predominant PIP isomer in thrombin-stimulated platelets to be PI 4-P. In this same report, however, considerable quantities of PI 3,4-P_2, in addition to PI 4,5-P_2, were observed in response to thrombin stimulation. Further research is required to identify which isomers of PIP and PIP_2 are increased following U44619 stimulation, as well as the potential functional importance of protein-tyrosine kinase in their formation.

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