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The diacylglycerol kinase inhibitor, R59949, potentiates secretion but not increased phosphorylation of a 47 kDalton protein in human platelets

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Receptor-stimulated hydrolysis of inositol phospholipids is now established as a major transmembrane signalling pathway generating two second messengers, inositol 1,4,5-trisphosphate (IP3), which releases intracellular Ca^{2+}, and 1,2-diacylglycerol (DG), which activates protein kinase C [1]. Activation of this pathway in human platelets e.g. following stimulation of thrombin, platelet activating factor, vasopressin or collagen receptors, leads to secretion of the contents of intracellular storage granules including 5-hydroxytryptamine (5-HT) and ATP [2, 3].

Nishizuka and others have shown that phorbol esters, which are potent activators of protein kinase C, produce a slow secretion of ATP or 5-HT in human platelets but that this response is markedly potentiated in the presence of raised intracellular Ca^{2+} [4, 5]. This suggests that receptor-stimulated phosphoinositide hydrolysis induces secretion by a synergistic interaction between PKC and Ca^{2+}. However, the relative importance of the role of PKC in secretion is uncertain since the activation of PKC is also associated with inhibition of certain platelet responses e.g. inhibition of phospholipase C [6] or stimulation of Ca^{2+} extrusion [7].

Recently, de Chaffoy de Courcelles *et al.* [8] have described a novel inhibitor of DG-kinase R59949. They have shown that R59949 inhibits DG-kinase in human platelets and potentiates 5-HT secretion induced by vasopressin. In the present study we have used this agent to investigate the role of PKC during 5-HT secretion induced by thrombin in human platelets and show that R59949 partially inhibits DG-kinase and potentiates secretion but that this is not associated with increased phosphorylation of a 47 kDa protein (identified as a protein of relative molecular mass 40–47 kDa and the major substrate for PKC [13]).

Methods

Platelets were obtained either from aspirin-free volunteers or from platelet concentrates, prepared from blood donated within the previous 24 hr to the Blood Transfusion Unit, John Radcliffe Hospital. Platelet-rich plasma was

prepared from 20 mM sodium citrate anticoagulated blood by centrifugation at 200 *g* for 20 min. 5-HT secretion, phosphatidic acid (PA) and protein phosphorylation were measured as previously described [6, 7, 9]. These methods are described briefly below.

Platelets were prelabelled with [^{3}H]5-hydroxytryptamine (10 μCi/20 mL) in platelet-rich plasma or platelet concentrate and incubated for 1 hr at 37°. They were then centrifuged in the presence of prostacyclin at 2000 *g* for 10 min and resuspended in a modified Tyrodes buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM NaH_2PO_4, 1 mM $MgCl_2$, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 5 mM glucose, 1 mM EGTA (ethyleneglycolbis (aminoethylether)tetraacetate), pH 7.3) at a concentration of 2×10^8/mL in the presence of indomethacin.

For protein phosphorylation and PA measurements platelet rich plasma was centrifuged in the presence of prostacyclin at 2000 *g* for 10 min and platelets resuspended in 1 mL of the above Tyrodes buffer and labelled with 2 mCi [^{32}P]orthophosphate at 37° for 60 min. They were then washed in the presence of prostacyclin and resuspended in Tyrodes buffer containing 10 μM indomethacin at a final concentration of 4×10^8 platelets/mL.

Platelet suspensions were prewarmed at 37° for 5 min before the addition of R59949 or its solvent, followed, after a variable incubation time, by stimulant. Reactions were stopped with 6% (v/v) glutaraldehyde in phosphate buffer for analysis of 5-HT secretion; with $CHCl_3$/methanol/HCl (50:100:1) for measurement of PA or with Laemmli buffer for analysis of protein phosphorylation.

Radioactive materials were purchased from New England Nuclear (Dupont). Thrombin, 1-oleoyl, 2-acetylglycerol (OAG) and dioctanoylglycerol (DC8) were from the Sigma Chemical Co. (St Louis, MO). R59949 (3-[2-[4-[bis(4- fluorophenyl)methylene]- 1- piperidinyl]ethyl]- 2,3-dihydro-2-thioxo-4(1*H*)-quinazolinone) was from Janssen (B-2430 Olen, Belgium). Prostacyclin was kindly donated by Wellcome Laboratories (Beckenham, Kent, U.K.). All other reagents were of analytical grade.

Results and Discussion

R59949 caused a small potentiation of thrombin-induced secretion of 5-HT. This effect was dose and time dependent and was evident for all sub-maximal concentrations of thrombin (Fig. 1A). Maximal potentiation of 5-HT secretion by R59949 was observed with a concentration of 3 μM (Fig. 1B) and a pre-incubation time of 10 min (Fig. 1C) and amounted to approximately 10–20% of the total 5-HT content within the platelet for an approximate EC_{50} concentration of thrombin (Fig. 1). No effect of the solvent used to prepare R59949 on thrombin-induced secretion was observed. These results are similar to those reported by de Chaffoy de Courcelles *et al.* [8] for R59949-potentiation of 5-HT secretion induced by vasopressin in human platelets; these authors also used a pre-incubation time of 10 min and a concentration of 3 μM R59949.

R59949 also caused a concentration-dependent inhibition of thrombin-induced formation of PA over a similar concentration range to that at which it potentiated secretion of 5-HT (Fig. 2). The effect of R59949 on unstimulated levels of PA formation showed no significant change over basal levels (0 ± 7%). Maximal inhibition of phosphatidic formation was observed at approximately 3 μM R59949; this concentration of R59949 reduced the formation of PA by thrombin by approximately 55% (Table 1). These results demonstrate that R59949 produces a similar degree of inhibition of thrombin-induced formation of PA to that seen by the prototype DG-kinase inhibitor R59022, although it is slightly more potent [10]. Further, this inhibition is of a similar order to that reported by de Chaffoy de Courcelles *et al.* [8] for the action of R59949 against vasopressin-induced formation of PA. However, we failed to confirm

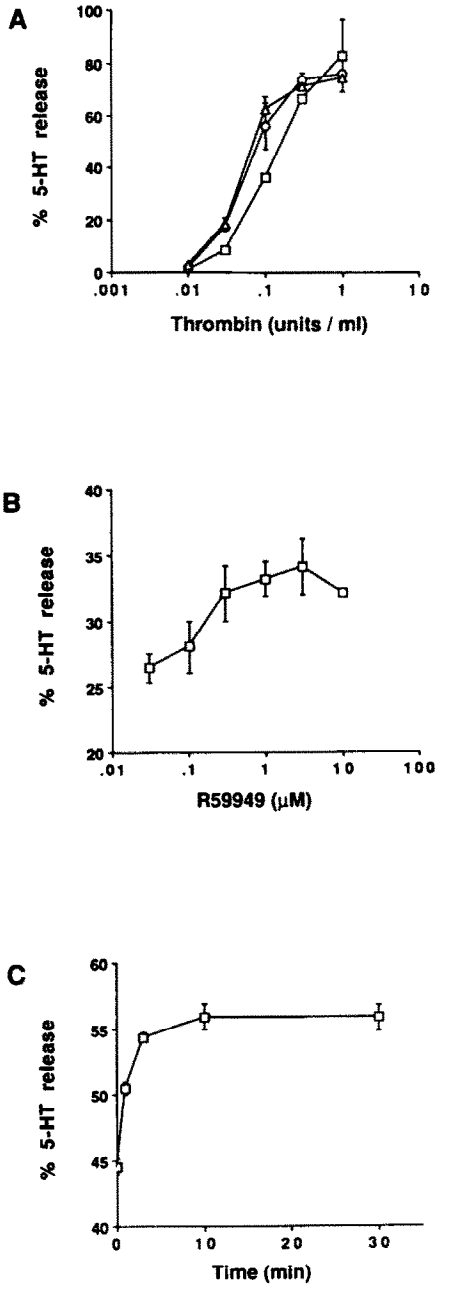

Fig. 1. (A) Effect of R59949 on [^{3}H]5-hydroxytryptamine secretion induced by thrombin. Human platelets were labelled with [^{3}H]5-HT as described in the Methods section and challenged with thrombin for 60 sec following pre-incubation with R59949 for 15 min. (□) Control, (○) 1 μM R59949, (△) 10 μM R59949. Results are shown as mean ± SEM from one experiment that is representative of two other similar experiments. (B) Concentration response curve for R59949 potentiation of thrombin-induced 5-HT release. Platelets were challenged with thrombin 0.1 unit/mL for 60 sec following pre-incubation with R59949 for 15 min. Results are shown as mean ± SEM from one experiment that is representative of two other similar experiments. (C) Time course of R59949-potentiation of 5-HT release in human platelets stimulated with thrombin. Platelets were preincubated with 1 μM R59949 for various times and challenged with thrombin (0.1 units/mL) for 60 sec.

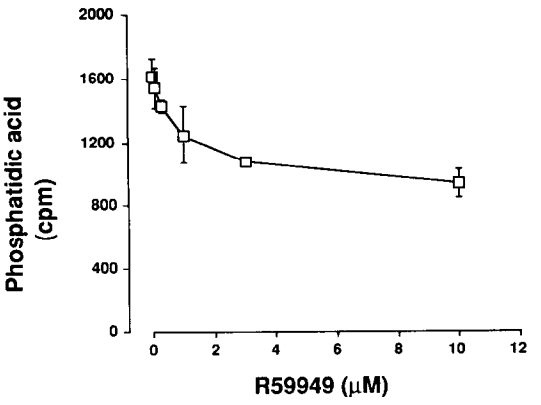

Fig. 2. Concentration–response curve for R59949 inhibition of thrombin-induced formation of [^{32}P]phosphatidic acid. Platelets, prelabelled with [^{32}P]orthophosphate, were challenged with thrombin (1 unit/mL) for 60 sec following pre-incubation with R59949 (0.1–10 μM) for 15 min. Results are shown from one experiment performed in triplicate; two other experiments gave similar results.

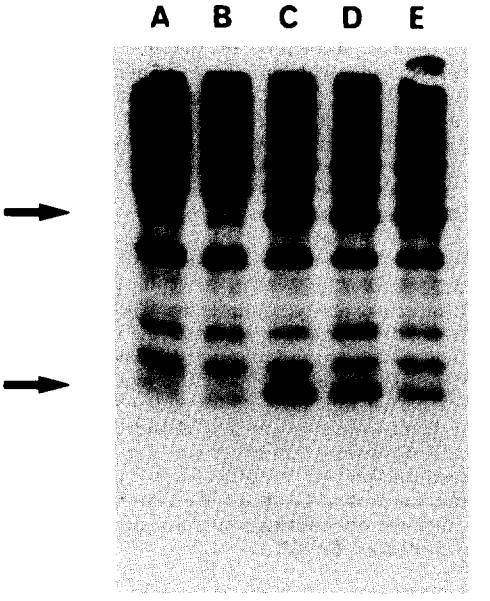

Fig. 3. Effect of R59949 on protein phosphorylation elicited by thrombin. An example autoradiogram of a one-dimensional gel from ^{32}P-labelled platelets challenged with 0.1 unit/mL thrombin (T) for 10 sec following pre-incubation with 3 μM R59949 for 15 min. Maximal phosphorylation of the 47 kDa protein by dioctanoylglycerol (DC8) is also shown. Four other experiments gave similar results. Lane A, basal; lane B, 3 μM R59949; lane C, 0.1 unit/mL thrombin; lane D, 3 μM R59949 + 0.1 unit/mL thrombin; lane E, 10 μM DC8. The arrows indicate the 47 kDa protein (upper) and the 20 kDa protein (lower).

the finding of de Chaffoy de Courcelles *et al.* [8] that R59949 inhibits completely the metabolism of OAG to OAG-PA. We obtained an approximate 55% inhibition of formation of OAG-PA from OAG with 10 μM R59949 (Table 1); a similar degree of inhibition was also obtained for the conversion of CD8 to DC8-PA (Table 1). Thus at a maximally effective concentration, R59949 produces a similar inhibition of the conversion of membrane-permeable diacylglycerols to PA to that seen with thrombin or vasopressin-induced formation of PA.

The above results for thrombin are similar to those obtained by de Chaffoy de Courcelles *et al.* [8] for the action of R59949 against vasopressin in human platelets. However, in marked contrast to the study of de Chaffoy de Courcelles *et al.* [8], we did not observe increased phosphorylation of a 47 kDa protein, an identified substrate for protein kinase C, in the presence of R59949. These results are exemplified in Fig. 3 for a sub-maximal concentration of thrombin in the presence of 3 μM R59949. Essentially similar results were seen with a range of concentrations of R59949, following longer incubations with thrombin or with sub-maximal concentrations of DC8. Further, when we investigated the action of R59949 on phosphorylation of the 47 kDa protein by the phorbol ester, phorbol dibutyrate (50 nM), we obtained a small inhibition of phosphorylation of the 47 kDa protein (not shown).

There is no clear explanation for the discrepancy between our results on phosphorylation and those of de Chaffoy de Courcelles *et al.* [8]. However, it is possible that, since R59949 was observed to inhibit 47 kDa phosphorylation by phorbol dibutyrate, R59949 may have two competing actions on PKC: inhibition through an unknown mechanism and potentiation as a consequence of inhibition of DG-kinase (thereby leading to increased levels of DG). These effects may therefore have cancelled each other out in the present study. The results therefore demonstrate that R59949 does not appear to offer a major advance on the earlier inhibitor of DG-kinase, R59022 [10, 11]. Although

Table 1. Inhibition of phosphatidic acid formation by R59949

		Phosphatidic acid*		
		–	+R59949	
Thrombin	1 unit/mL	1648 ± 105	735 ± 82	(55.5 ± 4.9%)
DC8	10 μM	1607 ± 63	656 ± 35	(59.2 ± 2.1%)
OAG	50 μM	2656 ± 360	1215 ± 222	(54.3 ± 8.3%)

Results are dpm values of mean (±SEM) increase in phosphatidic acid (PA) above basal in the absence or presence of R59949 (3–10 μM). The results are from one experiment performed in quadruplicate, two other experiments yielded similar results. The percentage values on the right hand side of the table show the degree of inhibition of PA formation by R59949.

* PA species are as follows: thrombin—endogenous phosphatidic acid; dioctanoylglycerol (DC8)—DC8-PA; 1-oleoyl,2-acetyl-glycerol (OAG)—OAG-PA.

R59949 has a slightly greater potency than R59022, it produces a similar, *incomplete* inhibition of PA formation following challenge with thrombin or membrane-permeable diacylglycerols and this is not associated with increased phosphorylation of a substrate for PKC.

The study therefore offers no explanation for the potentiation of thrombin-induced secretion by R59949. However, there are a number of substrates for PKC in platelets, the phosphorylation of which cannot be readily quantified on a one-dimensional gel e.g. [12]. It is possible that phosphorylation of one of these is potentiated by R59949 and that this protein is involved in secretion. (The role of the 47 kDa protein in platelets is unclear and no direct evidence is available to suggest that it plays a role in secretion.)

In summary, we have investigated the action of a novel inhibitor of DG-kinase, R59949. This agent was found to produce partial inhibition of formation of phosphatidic acid in human platelets challenged with thrombin, DC8 or OAG. However, this effect was not associated with enhanced phosphorylation of a 47 kDa protein, a known substrate for protein kinase C. We therefore believe that this compound does not represent a major advance on its earlier prototype, R59022.

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Assessment of the drug inhibitor specificity of the human liver 4-methylumbelliferone UDP-glucuronosyltransferase activity

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Conjugation with glucuronic acid, a process catalysed by the enzyme UDP-glucuronosyltransferase (UDPGT), is responsible for the metabolism of many clinically used drugs. It is now well established that UDPGT exists as a multi-gene family in the rat and that individual UDPGT rat liver isozymes tend to differ in terms of regulation and xenobiotic substrate specificity [1]. The multiplicity of human liver UDPGT is also now accepted on the basis of results from microsomal kinetic and inhibitor studies [2–6], the cloning of UDPGT cDNAs [7–9] and the isolation of purified enzymes [10]. However, despite the recognition of the heterogeneity of human liver UDPGT, little is known about isozyme substrate specificity, particularly with respect to therapeutic drugs.

One of the human liver microsomal UDPGT activities characterized to date [4] is that associated with the glucuronidation of the xenobiotic substrate 4-methylumbelliferone (4MU). Available evidence suggests the 4MU-UDPGT activity comprises at least two closely related isozymes with similar kinetic properties and broadly comparable chemical substrate specificities [4, 8, 10]. To determine the possible importance of the isozymes comprising