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SPACIAL ISOLATION OF PROTEIN KINASE C ACTIVATION IN THROMBIN STIMULATED HUMAN PLATELETS

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SUMMARY: Thrombin stimulation of human platelets is associated with turnover of inositol phospholipids, mobilization of intracellular Ca^{2+} stores, and activation of protein kinase C. However, within 5 minutes, the thrombin receptor desensitizes, but can be re-coupled to its effectors by stimulation of α_2-adrenergic receptors (Crouch and Lapetina, J. Biol. Chem. 263, 3363- 3371, 1988). This effect of epinephrine was found to be inhibited by preincubation of platelets with phorbol ester, suggesting that protein kinase C was inhibitory. However, since thrombin also activated protein kinase C and epinephrine was active following thrombin stimulation of platelets, this implied that thrombin activation of protein kinase C may have been spacially isolated near the thrombin receptor and could not inactivate α_2-receptor activity. In the present paper, we have tested this possibility, and we present evidence which strongly favours the possibility that protein kinase C activation by receptors induces its local translocation to the cell membrane.

Protein kinase C is critically important in the activation of cells by agonist occupation of a variety of receptors (1). This is particularly true for the class of receptors that stimulate inositol phospholipid turnover (2,3). The activation of platelet receptors, such as those for thrombin, platelet activating factor (PAF), and vasopressin, induces the hydrolysis of phosphatidylinositol-4,5-bisphosphate by phospholipase C (4). The result is the generation of inositol 1,4,5- trisphosphate (IP3) (5,6) and 1,2-diacylglycerol (DAG) (7), which mobilizes intracellular Ca^{2+} stores

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Abbreviations: IP3, inositol 1,4,5-trisphosphate; DAG, 1,2-diacylglycerol; D_iC_8, 1,2-dioctanoyl-sn-glycerol; PAF, platelet activating factor; PDBu, phorbol 12,13-dibutyrate.

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(3,8) and activates protein kinase C (1), respectively. These initially separate arms of the signalling pathway converge at three points at least. Firstly, DAG reduces the Ca^{2+} requirement for activation of protein kinase C (1). Secondly, there is a more downstream synergism between protein kinase C stimulation and elevation of the cytosolic Ca^{2+} level in which the cellular response, such as platelet secretion, is greater when both occur in parallel than individually (1,4). Thirdly, the actions of protein kinase C in signal transduction are associated with a Ca^{2+}-stimulated translocation of this enzyme to the plasma membrane where the enzyme is activated by DAG (1,2).

This scheme suggests that protein kinase C could conceivably be activated at localized sites at the plasma membrane. Two ways in which this could occur are through the local, receptor-induced DAG production and/or Ca^{2+} mobilization, which could then induce a directed translocation and activation of protein kinase C.

Results from a previous study from this laboratory (9) suggested that such a local activation of protein kinase C may have been occurring. The thrombin-induced mobilization of Ca^{2+} was found to be transient because of an agonist-induced desensitization of the thrombin receptor. However, the thrombin receptor (9) could be recoupled to Ca^{2+} mobilization and phospholipase C by activation of α_2-adrenergic receptors with epinephrine (9). The α-adrenergic receptor is not itself coupled to phospholipase C (9,10). The ability of epinephrine to reactivate thrombin receptors was totally inhibited by pretreating platelets with phorbol ester (9), implying that protein kinase C was phosphorylating and inactivating a component of the α_2-adrenergic receptor coupling system. However, thrombin receptors also activate protein kinase C, but epinephrine was effective after thrombin stimulation. This implied that perhaps protein kinase C activation in response to thrombin was a local event in the cell, but that phorbol ester produced an indiscriminant translocation and activation of this enzyme.

EXPERIMENTAL PROCEDURES

Platelet preparation, measurement of Ca^{2+} mobilization, and protein phosphorylation was as described before (9,11). All experiments are representative of at least two other similar experiments. 1,2-Dioctanoyl-sn-glycerol (D_iC_8) was from Novabiochem.

RESULTS

Phorbol ester and synthetic 1,2-diacylglycerol both desensitize epinephrine action - lack of effect of hormone agonists. As has been shown previously (9,11), activation of human platelets by α-thrombin (10 nM) produces a rapid mobilization of intracellular Ca^{2+} stores (fig 1). This is followed by a

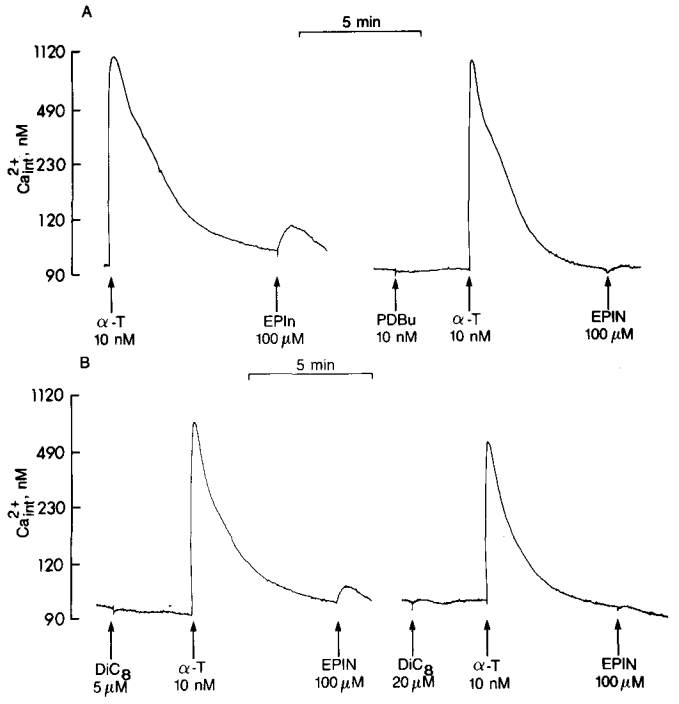

Figure 1 Phorbol 12,13-dibutyrate and 1,2-dioctanoyl-sn-glycerol inhibit epinephrine action on human platelets. Human platelets were loaded with a fluorescent indicator, Indo-1, and the intracellular Ca^{2+} concentration was measured in a fluorescence spectrophotometer. When used, phorbol 12,13-dibutyrate and 1,2-dioctanoyl-sn-glycerol were added 3 min before α-thrombin. α-T, α-thrombin; EPIN, epinephrine; PDBu, phorbol 12,13-dibutyrate; DiC8, 1,2-dioctanoyl-sn-glycerol.

decline of the Ca^{2+} response, which is due to homologous and heterologous desensitization processes (9,11). However, epinephrine will recouple the thrombin receptor to Ca^{2+} mobilization (fig 1A). Preincubating platelets for 3 min with phorbol 12,13-dibutyrate (PDBu) (10 nM) totally inhibited this effect of epinephrine (fig 1A). Similar effects were seen when platelets were preincubated with 1,2-dioctanoyl-sn-glycerol (DiC8), (fig 1 B). The inhibitory effects of DiC8 were half maximal at 5 μM and maximal at 20 μM (fig 1B).

In contrast, if platelets were challenged with endoperoxide analog U44069 (10 μM) and platelet activating factor (PAF, 1 μM) before (not shown) or following thrombin addition, epinephrine was still able to activate a subsequent release of intracellular Ca^{2+} stores (fig 2).

Protein phosphorylation in response to α-thrombin, endoperoxide U44069, PDBu and DiC8. A comparison of the abilities to activate protein kinase C (as shown by phosphorylation of the 47 kDa platelet substrate) showed that α-thrombin (10 nM) and endoperoxide U44069 (10 μM) were much more effective

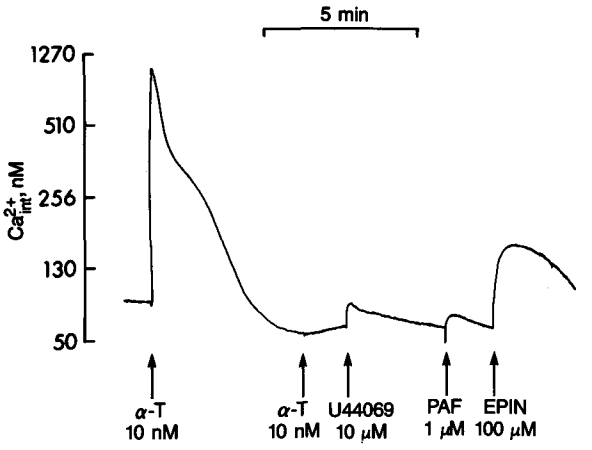

Figure 2 The effect of endoperoxide U44069 and platelet activating factor on epinephrine action. Intracellular Ca^{2+} concentration was measured using Indo-1. Agonists were added at the indicated times. α-T, α-thrombin; U44069, endoperoxide analog U44069; PAF, platelet activating factor; EPIN, epinephrine.

than PDBu (10 nM) (fig 3). Fig 4 compares the abilities of α-thrombin, PAF, endoperoxide U44069, and vasopressin to activate protein kinase C.

We also examined a more extended time course of phosphorylation in response to α-thrombin (10 nM), PDBu (10 nM), and DiC8 (5 and 20 μM)

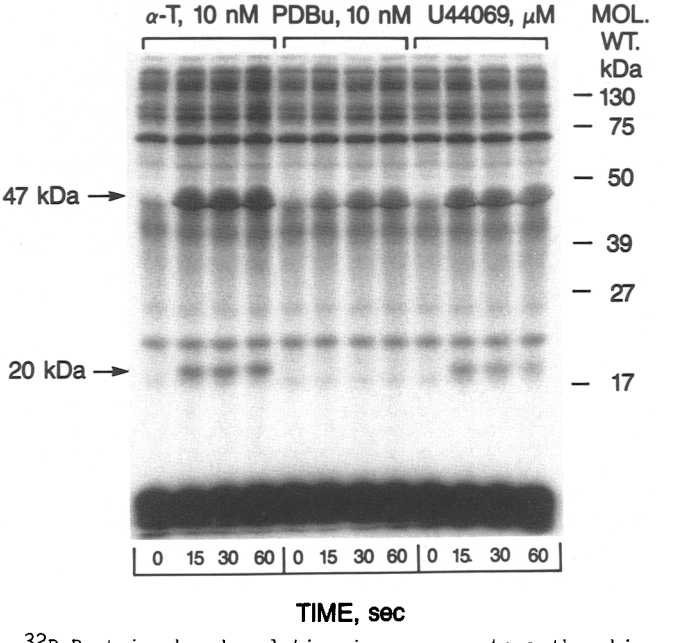

Figure 3 ^{32}P-Protein phosphorylation in response to α-thrombin, phorbol ester and endoperoxide U44069. ^{32}P-Labelled platelets were activated with either α-thrombin (α-T, 10 nM), phorbol 12,13-dibutyrate (PDBu, 10 nM), or endoperoxide U44069 (U44069, 10 μM) for the indicated times. Reactions were stopped by adding aliquots to SDS-PAGE sample buffer, and the proteins were then separated on SDS-PAGE and visualized by autoradiography. Positions of molecular weight standards are indicated.

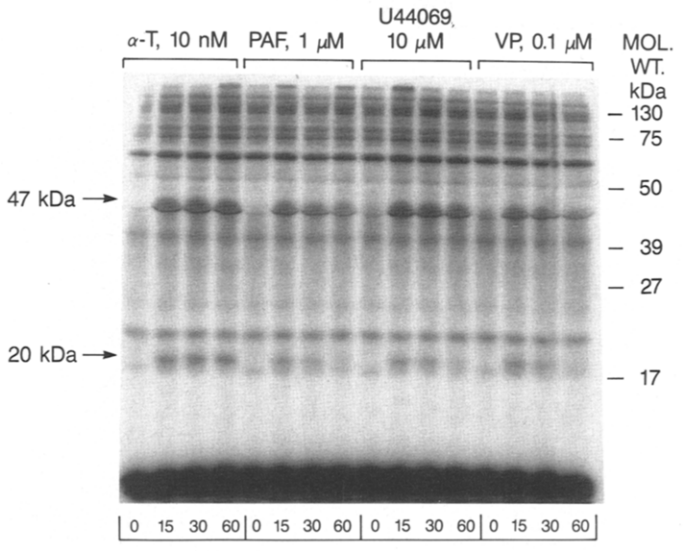

Figure 4 ^{32}P-Protein phosphorylation in response to α-thrombin, platelet activating factor, endoperoxide U44069 and vasopressin. ^{32}P-Labelled platelets were activated with either α-thrombin (α-T, 10 nM), platelet activating factor (PAF, 1 μM), endoperoxide U44069 (U44069, 10 μM) or vasopresin (VP, 0.1 μM) for the indicated times. Reactions were stopped by adding aliquots to SDS-PAGE sample buffer, and the proteins were then separated on SDS-PAGE and visualized by autoradiography. Positions of molecular weight standards are indicated.

(fig 5). Again, α-thrombin was the most potent agonist. Protein kinase C activation in response to PDBu was weaker but was sustained for up to 8 min after addition. DiC8 displayed a biphasic protein kinase C activation, at both 5 and 20 μM concentrations (fig 5). Maximal phosphorylation in response to 20 μM DiC8 was the same as that found in response to PDBu, whereas 5 μM DiC8 induced very little phosphorylation, and after 8 min there was no difference in 47 kDa protein phosphorylation from that found in unactivated samples (fig 5).

DISCUSSION

The steps involved in hormonal activation of protein kinase C have largely been elucidated (1,2,12). They include the receptor-induced degradation of inositol phospholipids (1,3,4) (and perhaps other phospholipids, 13) to form DAG. A rise in the cytosolic Ca^{2+} level then induces the translocation of protein kinase C to the cell membrane where DAG can bind and activate the enzyme (1,2). As discussed in the Introduction, the possibility exists that protein kinase C could be activated in local domains of the cell, based on selective translocation (such as by local increases in Ca^{2+} concentration) or on selective activation (due to localized production of DAG).

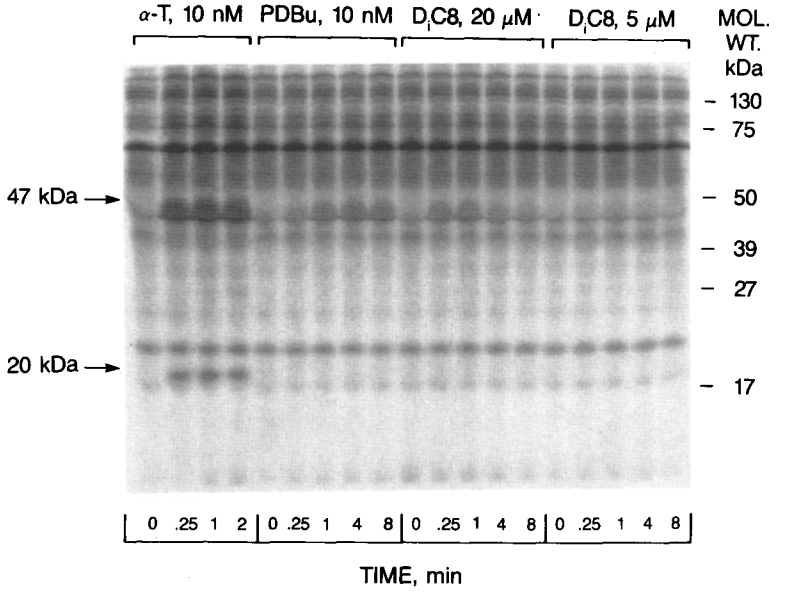

Figure 5 Time course of protein phosphorylation in response to α-thrombin, phorbol ester and 1,2-dioctanoyl-sn-glycerol. ^{32}P-Labelled platelets were activated with either α-thrombin (α-T, 10 nM), phorbol 12,13-dibutyrate (PDBu, 10 nM), or 1,2-dioctanoyl-sn-glycerol (D_iC_8, 20 or 5 μM)for the indicated times. Reactions were stopped by adding aliquots to SDS-PAGE sample buffer, and the proteins were then separated on SDS-PAGE and visualized by autoradiography. Positions of molecular weight standards are indicated.

We have presented a series of findings that suggest that protein kinase C is activated in localized domains of the thrombin-stimulated human platelet. Epinephrine acting on α_2-adrenergic receptors is able to reverse the desensitization of the thrombin receptor, as shown in a previous study (9). This effect was extremely sensitive to phorbol esters (9). The ability of both PDBu and exogenous DiC8 to inhibit epinephrine action strongly implicates protein kinase C activation in this inhibition, rather than another nonspecific mechanism. In addition, a protein kinase C inhibitor, staurosporine, can reverse this effect of PDBu (9,11).

Our present experiments were done because thrombin is itself a potent activator of protein kinase C. That is, how could the effects of epinephrine be inhibited by phorbol esters but not by thrombin. The obvious explanation, which we have explored, is that the protein kinase C, which was activated by thrombin, was not accessible to the α_2-adrenergic receptor, whereas it was in response to phorbol ester .

Our results show that α-thrombin, at the concentration used, was a far better stimulator of protein kinase C than PDBu. However, preincubation of platelets with PDBu was effective at inhibiting epinephrine action. One way in which this could be explained without invoking spacial isolation of protein kinase C activation is that in response to α-thrombin, after 5 min

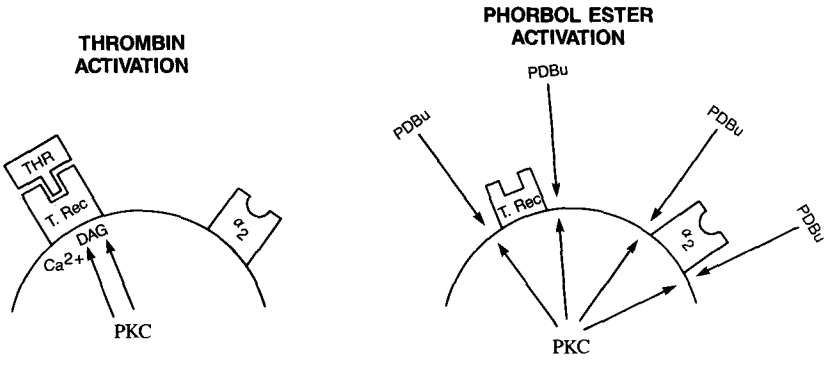

Figure 6 Spacial isolation of protein kinase C activation in thrombin stimulated human platelets. For details see Discussion. THR, thrombin; T. Rec., thrombin receptor; DAG, 1,2-diacylglycerol; PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; α_2, α_2-adrenergic receptor.

of stimulation, the receptor has desensitized and protein kinase C activation has returned to basal levels. This could then allow epinephrine action. Because of platelet aggregation, we could not study protein phosphorylation at these late time points in response to thrombin. However, we have answered the question in two other ways.

Firstly, preincubating platelets with DiC8 inhibited subsequent epinephrine action. However, protein phosphorylation in response to DiC8 was biphasic (14 and fig 5), and the level of protein kinase C activation after the same time interval was either very low (20 μM DiC8) or at basal levels (5 μM DiC8). Therefore, inhibition of epinephrine action by DiC8 did not require the sustained activation of protein kinase C, and so a biphasic activation of this enzyme by α-thrombin could not explain a lack of inhibitory action.

Secondly, following α-thrombin desensitization, we challenged platelets with endoperoxide U44069 and PAF before adding epinephrine. Both of these agonists activate protein kinase C, and both were partially active for Ca^{2+} mobilization following the α-thrombin stimulation. However, the epinephrine effect was not diminished by pretreating platelets with these agonists. Thus, not only did α-thrombin-induced protein kinase C activation have no inhibitory effect on epinephrine action, but neither did that in response to endoperoxide U44069 or PAF.

We believe these results are strong evidence in favor of the suggestion that protein kinase C activation in response to α-thrombin, and also endoperoxide U44069 and PAF, is spacially isolated (Fig. 6). This isolation probably would be near the occupied receptors where phospholipase C is activated and DAG is produced (Fig. 6). The generalized desensitization of epinephrine action by PDBu and DiC8 is explained by the nonspecific delivery

of these protein kinase C activators to the plasma membrane and generalized translocation of protein kinase C to the membrane where it could interact with all hormone receptors (Fig. 6).

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