Volume 268, number 2, 400–404 FEBS 08636 August 1990

Minireview

The signal transduction induced by thrombin in human platelets

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Received 15 May 1990

The stimulation of human platelets by thrombin leads to the activation of phospholipases C and A_2, protein kinases, formation of 3-inositol phospholipids and mobilization of Ca^{2+}. These biochemical reactions closely parallel platelet shape change, granular secretion and aggregation. The membrane-bound transducers for the thrombin receptor seem to be the heterotrimeric G protein G_{i2} and the *ras*-related G protein rap 1-b. Phosphorylation of rap 1-b by the action of the cyclic AMP-dependent protein kinase seems to uncouple the thrombin receptor from phospholipases. This causes inhibition of the formation of second messenger molecules and the onset of physiological responses.

Heterotrimeric G protein; Small molecular weight G protein; Phospholipase C; Phospholipase A_2; 3-inositol phospholipid; Protein kinase A

1. INTRODUCTION

Thrombin is a potent agonist that elicits platelet physiological responses such as shape change, granular content secretion, and aggregation. The cellular transduction of thrombin action includes the activation of thrombin receptor-associated GTP-binding proteins and the subsequent stimulation of phospholipase C and phospholipase A_2 [1]. The products of these phospholipases generate specific biochemical messengers. For instance, phospholipase C causes the breakdown of inositol phospholipids and the production of 1,2-diacylglycerol, which activates protein kinase C [2], and inositol 1,4,5-trisphosphate [3], which mobilizes Ca^{2+} from the endoplasmic reticulum to the cytosol. The increase of cytosolic Ca^{2+} activates Ca^{2+}-dependent enzymes such as myosin light-chain kinase [1]. The stimulation of phospholipase A_2 also results in production of active molecules. The liberation of arachidonic acid from the 2-position of various phospholipids will generate arachidonate-derived metabolites through the cyclooxygenase and lipoxygenase activities [4]. These include the endoperoxides and thromboxanes, which can efficiently induce further platelet activation [4]. This chapter discusses fundamental aspects of the various signal transduction steps in the binding of thrombin to platelets.

2. MEMBRANE-BOUND TRANSDUCER

The membrane-bound transducer between the receptor and the phospholipase in the thrombin-induced platelet response seems to be a specific GTP-binding protein (G protein), which is functionally referred to as Gp [5]. The exact identity of this protein has not yet been determined. G proteins were originally described by their ability to affect adenylyl cyclase (G_s stimulates this enzyme while G_i inhibits) or cyclic GMP phosphodiesterase (G_t stimulates this enzyme in the visual system) [6–8]. These G proteins have a heterotrimeric structure consisting of α, β, and γ subunits. Other heterotrimeric G proteins, such as G_o and G_z, have been identified [6–8]. In each case the α subunit binds GTP and possesses an intrinsic GTPase activity. In addition, these α subunits can be specifically ADP-ribosylated by cholera toxin (α_s), by pertussis toxin (α_i, α_o), by both toxins (α_t), or not affected by toxin-induced ADP-ribosylation (α_z) [6–8]. Several species of $G_{s\alpha}$ and $G_{i\alpha}$ have been described [6–8]. For $G_{i\alpha}$, it is possible to distinguish three distinct proteins; these are referred to as $G_{i\alpha 1}$, $G_{i\alpha 2}$ and $G_{i\alpha 3}$ [9]. The three are about 90% identical and are localized in specific tissues [9]. The β subunit of the heterotrimeric G proteins corresponds to one or the other of closely related 35- and 36-kDa proteins, whereas the γ subunit is a low molecular mass protein (M_r 6000–10 000) [6–8]. It is known that the interaction of the receptor with a heterotrimeric G protein induces dissociation of the α subunit with release of the $\beta\gamma$ subunit [6–8]. Investigators have implicated G_i in the modulation of phospholipase C in those cases where pertussis toxin inhibits the agonist-induced stimulation of phospholipase C [9]. G_z has been postulated as a mediator for activation of phospholipase C through a pertussis toxin-insensitive pathway [9]. Moreover, a recent publication

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00145793/90/$3.50

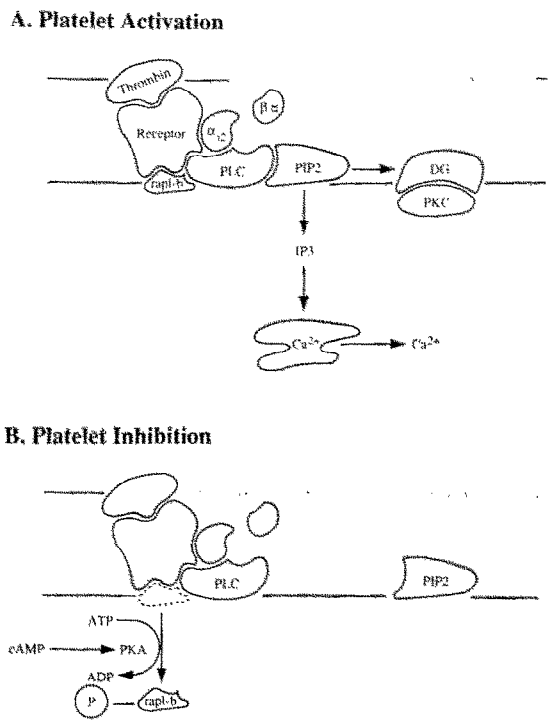

Fig. 1. Transmembrane signaling in platelets. (A) Activation of platelets. The interaction of thrombin with its platelet receptor results in activation of phospholipase C (PLC). The membrane-bound transducers that relate the message from the receptor to the PLC seem to be the heterotrimeric G protein G_{i2} (that contains the subunits α_{i2}, β and γ) and the low molecular mass *ras*-related G protein, rap1-b. The activation of the PLC induces the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), which causes the formation of two second messenger molecules. These are 1,2-diacylglycerol (DG), which activates protein kinase C (PKC), and inositol 1,4,5-trisphosphate (IP_3), which releases Ca^{2+} from the endoplasmic reticulum to the cytosol. (B) Inhibition of platelet responses. Increases of platelet cyclic AMP produce activation of protein kinase A (PKA), which phosphorylates the *ras*-related G protein, rap1-b. Phosphorylation of this protein causes its translocation to the cytosol. This leaves PLC without the ability to be activated by a receptor-coupled mechanism.

indicates that G_o is directly involved in the regulation of phospholipase C [10].

In platelets, thrombin produces the dissociation of $G_{i\alpha 2}$ from $G_{\beta\alpha}$ [11–13]. $G_{i\alpha 2}$ is thus involved in the transduction of thrombin stimulation of platelets [11–13], which is depicted in Fig. 1. However, it is not known if the released $G_{i\alpha 2}$ directly affects phospholipase C. We have described a *ras*-related G protein, rap1-b, that may exert a tonic effect on phospholipase C [14–18]. This protein will be discussed in the last section of this chapter. It has also been suggested that an additional factor – one related to the proteolytic action of thrombin – is needed for actual activation of phospholipase C [19]. This proteolytic action might be exerted directly on phospholipase C, on the receptor for thrombin, or on another protein (perhaps the receptor-associated G protein) that is intimately related to membrane-bound phospholipase C. Observations that epinephrine, which dissociates $G_{i\alpha 2}$ from $\beta\gamma$, does not stimulate phospholipase C in human platelets [12] suggest that $G_{i\alpha 2}$ does not directly stimulate phospholipase C. In summary, $G_{i\alpha 2}$ seems to be a membrane transducer of thrombin's effect on platelets. However, no specific effect of $G_{i\alpha 2}$ on phospholipase C is known. It is possible that an additional mechanism involving rap1-b and thrombin proteolytic activity is responsible for the stimulation of phospholipase C [19].

3. ACTIVATION OF INOSITIDE METABOLISM IN HUMAN PLATELETS

Thrombin can effectively and rapidly produce the activation of phospholipase C in human platelets [1]. This event is one of the earliest measurable reactions in the cascade of steps that follows thrombin action on human platelets [1]. The activation of phospholipase C causes the hydrolysis of the inositol phospholipids, i.e. phosphatidylinositol, phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-bisphosphate [3] (Fig. 2). Phosphatidylinositol 4,5-bisphosphate seems to be hydrolysed preferentially at the onset of thrombin action [20]. This hydrolysis causes the formation of 1,2-diacylglycerol and inositol 1,4,5-triphosphate (Fig. 2), both of which can be measured readily. The activation of protein kinase C by 1,2-diacylglycerol is shown by the massive phosphorylation of a 47-kDa protein [1]. Inositol 1,4,5-trisphosphate induces rapid mobilization of Ca^{2+} from the endoplasmic reticulum to the cytosol [1]. The effect of inositol 1,4,5-trisphosphate on the intracellular mobilization of Ca^{2+} can be studied in platelets that have been permeabilized with saponin [1].

The hydrolysis of phosphatidylinositol and phosphatidylinositol 4-monophosphate in platelets stimulated by thrombin further contributes to the formation of 1,2-diacylglycerol and the consequent stimulation of protein kinase C. Hydrolysis of these inositol phospholipids also produces formation of inositol 1-monophosphate and inositol 1,4-bisphosphate (Fig. 2).

The early hydrolysis of phosphatidylinositol 4,5-bisphosphate triggers the activation of the inositide kinases to replenish the pool of phosphatidylinositol 4,5-bisphosphate (Fig. 2). This was demonstrated by studies in which the increased labeling of phosphatidylinositol 4,5-bisphosphate was observed after its initial drop in thrombin-stimulated platelets [20]. The first enzyme involved in this process is phosphatidylinositol 4-kinase, which transfers a phosphate from ATP to the 4-position of the inositol part of the phosphatidylinositol molecule to form phosphatidylinositol 4-monophosphate. Subsequently, phosphatidylinositol 4-monophosphate 5-kinase phosphorylates phosphatidylinositol 4-monophosphate

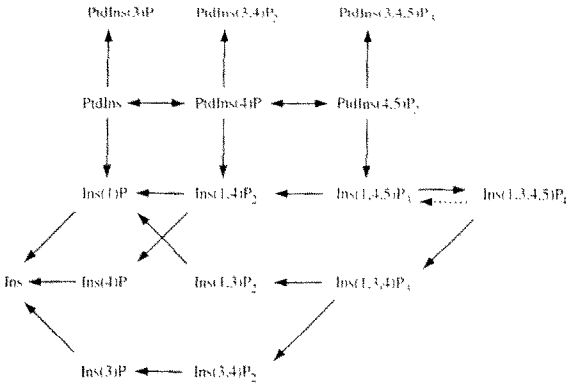

Fig. 2. Metabolism of inositides and inositol phosphates. Phosphatidylinositol (PtdIns) can be sequentially phosphorylated by specific kinases to phosphatidylinositol 4-monophosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2). The polyphosphoinositides can also be dephosphorylated by specific phosphomonoesterases. A specific inositide-phospholipase C (phosphoinositidase) can hydrolyze the inositol phospholipids to inositol 1-monophosphate (Ins1P), inositol 1,4-bisphosphate (Ins1,4P_2), and inositol 1,4,5-triphosphate (Ins1,4,5P_3). The three inositides can also be phosphorylated by a specific phosphatidylinositol 3-kinase to produce the respective 3-inositides: phosphatidylinositol 3-monophosphate (PtdIns3P), phosphatidylinositol 3,4-bisphosphate (PtdIns3,4P_2), and phosphatidylinositol 3,4,5-triphosphate (PtdIns3,4,5P_3). Inositol 1,4,5-trisphosphate (Ins1,4,5P_3) can be sequentially dephosphorylated to inositol 1,4-bisphosphate (Ins1,4P_2), inositol 4-monophosphate (Ins4P), and inositol (Ins). Ins1,4,5P_3 can alternatively be phosphorylated by a 3-kinase to inositol 1,3,4,5-tetrakisphosphate (Ins1,3,4,5P4), which, in turn, can be dephosphorylated to inositol 1,3,4-trisphosphate (Ins1,3,4P_3) or Ins1,4,5P_3. Ins1,3,4P_3 is dephosphorylated to inositol 1,3-bisphosphate (Ins1,3P_2) or inositol 3,4-bisphosphate (Ins3,4P_2). Ins1,3P_2 seems to be sequentially dephosphorylated to inositol 1-monophosphate (Ins1P) and Ins, whereas Ins3,4P_2 is dephosphorylated to inositol 3-monophosphate (Ins3P) and Ins.

to form phosphatidylinositol 4,5-bisphosphate. The interconversion of the inositol phospholipids and the derived inositol phosphates produced by phospholipase C is depicted in Fig. 2 [3]. A kinase, inositol 1,4,5-trisphosphate 5-kinase, can further phosphorylate inositol 1,4,5-trisphosphate to form inositol 1,3,4,5-tetrakisphosphate [3]. All the inositol phosphates shown in Fig. 2 are dephosphorylated by different inositol phosphatases leading to the formation of inositol as shown in Fig. 2 [3]. Inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate are dephosphorylated by the same enzyme, a 5-phosphatase [3]. Inositol 1,3,4,5-tetrakisphosphate has also been shown to be dephosphorylated to inositol 1,4,5-trisphosphate by a 3-phosphatase, which is activated by thrombin and phorbol ester [21].

4. THROMBIN PRODUCES NOVEL POLYPHOSPHOINOSITIDES, THE 3-INOSITOL PHOSPHOLIPIDS, IN HUMAN PLATELETS

Recent reports have documented phosphatidylinositol 3-kinase activity in different cells that is regulated by receptor occupation and results in phosphoinositides phosphorylated at the 3-position on the inositol ring (Fig. 2) [22]. Such inositol phospholipids may act as novel second messengers themselves since they are not substrates for phospholipase C [23]. The phosphatidylinositol 3-kinase in NIH-3T3 cells is specifically associated with middle T/pp60$^{c\text{-}src}$ protein from middle T-transformed cells as well as with antiphosphotyrosine immunoprecipitates from platelet-derived growth factor-stimulated fibroblasts [22]. Platelets contain abundant levels of a pp60$^{c\text{-}src}$-related protein-tyrosine kinase, which in human platelets is activated by thrombin treatment [24]. Thrombin stimulates the formation of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate in human platelets [25,26]. The level of phosphatidylinositol 3,4-bisphosphate is also increased by thrombin in human erythroleukemia cells that posses a number of platelet-like features [27]. These novel lipids appear to represent an as yet uncharacterized branch of the inositide cycle in activated platelets and may be involved in transmembrane signaling in activated platelets. In other proliferating cells, they have been implicated in transduction of the mitogenic signal. The role, if any, of pp60$^{c\text{-}src}$ in the formation of the 3-inositol phospholipids in platelets has yet to be elucidated.

5. ACTIVATION OF PHOSPHOLIPASE A_2

Thrombin is very active in releasing arachidonic acid from membrane phospholipids of platelets [1,4]. The primary enzymes responsible for this activation are phospholipase A_2 and, to a lesser degree, 1,2-diacylglycerol lipase [1,4]. Thrombin activation of platelets depends on arachidonic acid release only at low concentrations of thrombin; at higher doses the ability of thrombin to activate platelets is independent of arachidonic acid metabolites [1,4]. The most important arachidonic acid metabolites in platelet stimulation are endoperoxides and thromboxane A_2. These products are derived from the concerted action of cyclooxygenase and thromboxane synthase.

It has been widely accepted that Ca^{2+} mobilization – a consequence of phospholipase C activation – is a prerequisite for the activation of phospholipase A_2 [1,4]. However, there are indications that phospholipase A_2 is not under the control of the prevailing Ca^{2+} level in the intact cell [28]. Collagen has been shown to activate phospholipase A_2 independently of changes in the cellular Ca^{2+} concentration [29]. Also, the ability of α-thrombin to induce the release of Ca^{2+} stores does not parallel the liberation of arachidonic acid [28]. This suggests that phospholipase C activation and phospholipase A_2 activation are separate events in platelet activation. Also, the mechanism by which α-thrombin stimulated

phospholipase A_2 does not appear to be through dissociation of the inhibitory G protein, G_i, since α-thrombin decreases the pertussis toxin-induced ADP-ribosylation of α_i as much as does α-thrombin, but is much less effective than α-thrombin at inducing arachidonic acid liberation [28].

The regulation of phospholipase A_2 is not yet understood. It is possible that the concerted action of Ca^{2+}, protein kinase C, and G_i dissociation control phospholipase A_2 activation in platelets. However, it is possible to rule out the existence of a single controlling factor of arachidonic acid liberation in this cell.

6. INHIBITION OF PLATELET RESPONSES

It is widely known that the increase of platelet cyclic AMP (cAMP) inhibits all platelet responses to activating factors including activation of phospholipases C and A_2 [1]. The exact mechanism of this effect is not well understood but it is believed to be related to the phosphorylation of specific proteins by cAMP-dependent protein kinase [1]. The inhibition of thrombin-induced phospholipase C by cAMP is also observed in the human erythroleukemia (HEL) cell [17]. A specific protein is phosphorylated by the action of cAMP in both cells. This is a 22-kDa protein that binds [α-^{32}P]GTP on nitrocellulose blots and displays strong reactivity with the monoclonal antibody M90 [14–18]. This antibody recognizes an epitope between amino acids 107 and 130 of the H-*ras* p21 protein, which is one of the major GTP-binding regions of the protein. Elevation of intracellular cAMP levels in platelets and HEL cells by the prostacyclin analog iloprost results in a shift of the apparent molecular mass of the M90 immunoreactive band from 22 kDa to 24 kDa. This shift correlates with translocation of the modified 22 kDa protein from a particulate to a cytosolic fraction [14,15]. It is proposed that these changes in the mobility of the 22-kDa protein are the result of a covalent modification of the protein such as phosphorylation. Indeed, the treatment of ^{32}P-labeled platelets with iloprost results in the phosphorylation of a 22- to 24-kDa GTP-binding protein by cAMP-dependent protein kinase [14]. This phosphorylated protein can be immunoprecipitated with the monoclonal antibody M90 [14,15], suggesting that the 22-kDa translocated protein and the phosphorylated protein are identical.

Amino acid analysis of the peptides obtained by digestion of the 22-kDa protein with trypsin or *Staphylococcus aureus* V8 protease has shown that the phosphorylated protein is rap1-b [18]. Three rap proteins have so far been identified and they all are about 50% identical with *ras* [30,31]. They are extensively homologous with *ras* at the GTP-binding region and the effector domain. They also contain a carboxyl-terminal CAAX motif that is thought to be essential for processing and membrane localization. Rap1-a (also called smg-21 [32] or Krev-1 [33]) has been shown to reverse the transformed phenotype of *ras*-transformed cells [33]. Rap1-b is the major substrate for phosphorylation by cyclic AMP-dependent protein kinase in platelets and human erythroleukemia cells [18]. Rap2 is membrane-bound and is isoprenylated (unpublished results). Since cAMP inhibits phospholipase C, it is possible that the phosphorylation and translocation of rap1-b has a significant role in the regulation of phospholipase C (and or phospholipase A_2). The unphosphorylated rap1-b may be closely associated with phospholipase C (Fig. 1). Upon phosphorylation and translocation of rap1-b, phospholipase C may lose the ability to be stimulated by thrombin or other agonists (Fig. 1). The confirmation of this hypothesis awaits further investigation.

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