

Regulation of Hormone-Induced Ca^{2+} Mobilization in the Human Platelets

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α -Thrombin, γ -thrombin, and platelet-activating factor each stimulated the mobilization of intracellular Ca^{2+} stores in aspirin-treated human platelets. This was followed by desensitization of the receptors, as shown by the return of the Ca^{2+} level to basal values and by the fact that a subsequent addition of a second different agonist, but not the same agonist, could again elicit a response. Epinephrine, acting on α_2 -adrenergic receptors, was by itself ineffective at mobilizing Ca^{2+} stores. However, when added after the thrombin-induced response, epinephrine could evoke a considerable release of Ca^{2+} from cellular stores. This appeared to be due to epinephrine recoupling thrombin receptors to phospholipase C. In support of this, epinephrine was able to induce the formation of inositol triphosphate when added after the response to thrombin had also become desensitized. Alone, epinephrine was without effect. Pre-activation of protein kinase C with the phorbol ester abolished these effects of epinephrine, suggesting that epinephrine was working by activating a protein which could be inactivated by phosphorylation. Our current work is to characterize this protein that may be a member of the G_i , GTP-binding protein family.

Introduction

In many mammalian cell types, stimulation of specific receptors by agonists is accompanied by a nonvoltage-regulated mobilization of intracellular Ca^{2+} stores. This results in the elevation of the cytosolic Ca^{2+} concentration, which can evoke cellular responses such as in the platelet, secretion, shape change, and aggregation. The Ca^{2+} appears to originate from specific, nonmitochondrial sites in the cell, probably the endoplasmic reticulum or the platelet-dense tubular system.

The mechanism by which agonists cause a receptor-mediated Ca^{2+} -mobilization has been extensively studied over recent years. It appears that the transduction mechanism of occupied receptors is the phospholipase C-catalyzed hydrolysis of inositol phospholipid, specifically, phosphatidylinositol-4,5-bisphosphate (PIP₂). The immediate products of this reaction are diacylglycerol (DAG), which can activate protein kinase C (1) and inositol trisphosphate (IP₃), which will mobilize the hormone-sensitive Ca^{2+} stores when applied to permeabilized cells (2).

Therefore, the connection between receptor and Ca^{2+} store mobilization has been quite convincingly established. The questions that we wanted to address

were a) what are the kinetics of Ca^{2+} mobilization in the platelet; b) did these correlate with changes in the IP₃ levels; and c) where in the chain of events following the agonist's receptor occupation is the release of Ca^{2+} modulated or controlled? We have discussed these points elsewhere (3).

Platelet Ca^{2+} Responses to Agonists

We chose three different agonists: α -thrombin, γ -thrombin, and the platelet-activating factor (PAF) to study Ca^{2+} mobilization in the platelet. Each of these agents was able to mobilize Ca^{2+} from intracellular Ca^{2+} stores, but to ranging degrees and rates (Fig. 1). α -thrombin was most effective, whereas γ -thrombin produced a smaller and slower response. PAF produced a rapid Ca^{2+} signal, but had a smaller magnitude than that of γ -thrombin.

Each of these responses was quite transient, despite the continued presence of the agonist; after the return to baseline levels, the cytosolic Ca^{2+} level could not be reelevated by a second addition of the same agonist at the same concentration (Fig. 1). Thus, the loss of response was not due to degradation of the agonist and could not be explained by the agonists fully depleting the Ca^{2+} stores, since PAF was a much less effective agonist than α - or γ -thrombin. The Ca^{2+} response to PAF returned most quickly to basal levels and could not be restimulated with PAF.

These responses appeared to be a desensitization of the receptors or of a post-receptor mechanism. To determine which of these two possibilities was more

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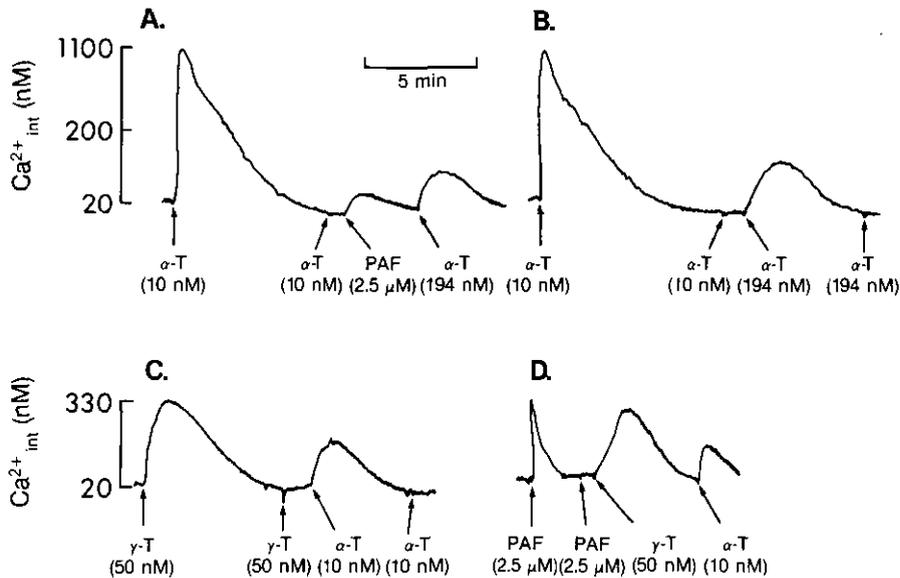


FIGURE 1. The effect of α -thrombin, γ -thrombin and platelet activating factor on intracellular Ca^{2+} mobilization in washed human platelets: homologous desensitization of each agonist by prolonged activation. Washed human platelets were treated with aspirin and then incubated for 45 min with 5 μM Indo-1 AM. After washing away the free Indo-1, platelets were resuspended in buffer, aliquots of platelets (2 mL) were placed into plastic cuvettes, and test agents added. Changes in fluorescence were monitored with a fluorimeter. The responses shown were highly reproducible with different donors. α -thrombin, α -T; γ -thrombin, γ -T; platelet activating factor, PAF; epinephrine, EPIN. For details see Crouch and Lapetina (3).

likely, the ability of a second agonist to elicit the release of Ca^{2+} stores was examined. After the α - or γ -thrombin induced Ca^{2+} release was complete, the cytosolic Ca^{2+} concentration had returned to basal levels, and the addition of the same agonist at the same concentration was shown to have no effect. PAF was still able to induce a small but significant release of Ca^{2+} stores (Fig. 1). The smaller response to PAF after the thrombin rather than to PAF alone is probably because the thrombin has already depleted much of the Ca^{2+} store, and the PAF can act to release only a much-reduced pool of Ca^{2+} .

Thrombin Receptor Desensitization

Because thrombin and PAF are both thought to couple through the same second messenger system (i.e., phospholipase C), this suggests that the desensitization observed is homologous and occurs at the receptor-effector site rather than a post-receptor site. It has been shown previously (4) that activation of protein kinase C, which occurs during stimulation of platelets with thrombin or PAF, is able to increase the degradation of cytosolic IP_3 by elevating IP_3 phosphatase activity. This could decrease the receptor-activated Ca^{2+} release. However, the activation of protein kinase C does not appear to be the mechanism of the desensitization observed here since this would cause a heterologous desensitization. The desensitization is better explained by being related to the generation of the Ca^{2+} -mobilizing signal (i.e., IP_3) rather than to the fate of IP_3 once it formed.

Thrombin Effects Are Restored by Epinephrine

To examine this effect further, we have used epinephrine, which is able to potentiate the action of many platelet agonists without having a direct effect on platelet responses. Epinephrine acts via α_2 -adrenergic receptors, since yohimbine was found to totally inhibit its actions. We found that epinephrine alone had no effect on platelet Ca^{2+} levels. However, when added to platelets previously desensitized to thrombin, epinephrine could elicit a relatively large mobilization of Ca^{2+} stores (Fig. 2). Thus, epinephrine was able to resensitize the thrombin receptor to the generation of a Ca^{2+} signal.

Phospholipase C Activation by Thrombin

In supporting the role of IP_3 in mobilizing intracellular Ca^{2+} stores, the desensitization of the thrombin-induced Ca^{2+} release and resensitization by epinephrine were paralleled by desensitization and resensitization of the α -thrombin-induced IP_3 formation (Fig. 3). This showed us that the thrombin receptor did desensitize at the level of receptor activation of phospholipase C and hydrolysis of PIP_2 , but that the α_2 -adrenergic receptor could resensitize thrombin receptor coupling. Those effects were able to fully explain our Ca^{2+} data.

Down-Regulation by Protein Kinase C

The next part of our study was to try to assess what caused the homologous desensitizations. Since each desensitization was coupled to specific receptors but showed similar characteristics, it was likely that the agonists induced a local activation of a second messenger, which was itself responsible for the receptor inactivation. It is known from studies of other cell types that protein kinase C activation can be inhibitory to hormone-induced phospholipase C. In the platelet, diacylglycerol, the endogenous protein kinase C activator, is rapidly converted to phosphatidic acid. Thus, diacylglycerol may well be produced as a local event and also locally activate protein kinase C near the occupied receptors.

When we treated platelets with the protein kinase C-activating phorbol ester, phorbol-12,13-dibutyrate (Pdbu), we found a reduction in the thrombin-induced release of Ca²⁺ stores and an abolition of resensitization of thrombin action by epinephrine (Fig. 4). This effect of Pdbu was half maximal at 1 nM and occurred within 30 sec, with both parameters being consistent with a specific activation of protein kinase C. From these results, we suggest that a local elevation in the DAG near the occupied receptor causes a local activation and translocation of protein kinase C so that only the occupied receptor is desensitized.

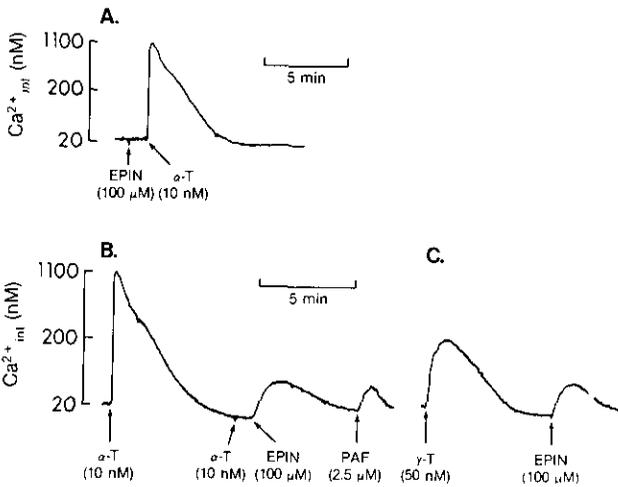


FIGURE 2. Epinephrine-induced resensitization of α -thrombin-stimulated Ca²⁺ mobilization is mediated by α_2 -adrenergic receptors. Receptor-induced release of intracellular Ca²⁺ stores was measured using Indo-1 AM-loaded human platelets, as described in Figure 1. For details see Crouch and Lapetina (3).

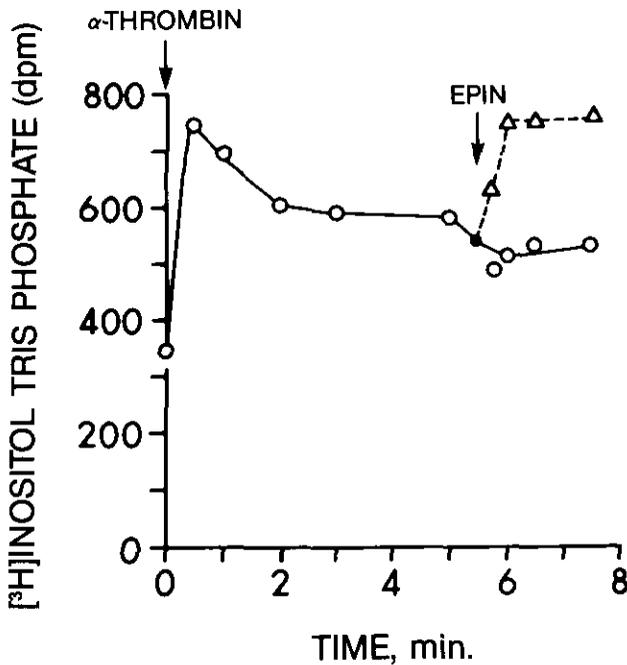


FIGURE 3. α -Thrombin-induced inositol trisphosphate formation is desensitized by prolonged stimulation and reversed by epinephrine. Inositol phospholipids of aspirin-treated, washed human platelets were labeled by an incubation of cells with *myo*-[2-³H]inositol. Stimulation of these cells with α -thrombin (α -T, 10 nM, $t = 0$) resulted in formation of inositol trisphosphate (IP₃). After 5.5 min, epinephrine (EPIN, 100 μ M) or buffer was added and further samples were assayed for the formation of inositol trisphosphates. For details see Crouch and Lapetina (3).

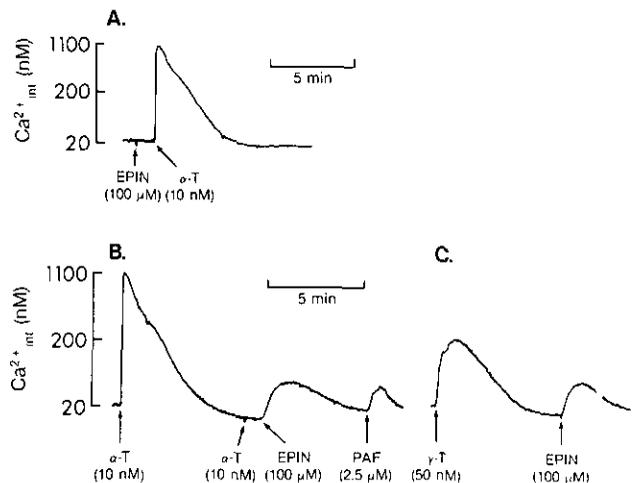


FIGURE 4. Phorbol esters reduce α -thrombin-induced mobilization of intracellular Ca²⁺ stores and abolish resensitization of the desensitized thrombin receptor by epinephrine. The release of intracellular Ca²⁺ stores by agonists was measured as detailed in Figure 1. Phorbol 12,13-dibutyrate (Pdbu) was added either 3 min before α -thrombin (as shown) or after α -thrombin, and 60 sec before epinephrine (not presented). For details see Crouch and Lapetina (3).

Conclusion

We believe that receptor desensitization in the human platelet represents an important negative feedback system in controlling platelet responses, including the mobilization of intracellular Ca^{2+} stores and activation of phospholipase C. This desensitization appears to be homologous and mediated by hormone-induced activation of protein kinase C. Our current work (3) implicates the inhibitory GTP-binding protein, G_i , as the substrate of protein kinase C involved in receptor-phospholipase C coupling.

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