Rapid effects of phorbol ester on platelet shape change, cytoskeleton and calcium transient

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Platelets are rapidly activated by several agonists. When phorbol 12-myristate 13-acetate (PMA) was added to washed platelet suspensions 10 s prior to either thrombin or ADP, it caused a dose-dependent inhibition of shape change correlated with decreased myosin association with the cytoskeleton and with inhibition of the calcium transient measured in fura-2-loaded platelets. PMA added 5–10 s after agonists did not reverse shape change or the association of myosin with the cytoskeleton, but markedly increased the rate at which the calcium signal returned to the baseline. The analogue, 4α-phorbol didecanoate did not cause these effects. Our results suggest that one effect of C-kinase activation is to provide negative feedback in sequential responses.

Phorbol ester *Protein kinase C* *(Blood platelet)* *Ca^{2+} transient* *Myosin* *Cytoskeleton*

1. INTRODUCTION

Platelet stimulation by agonists is followed in seconds by shape change [1]. A rise in free calcium is thought to be the triggering mechanism [2]. The free Ca^{2+} is most probably mobilized by *myo*-inositol-1,4,5-triphosphate (IP_3), released from phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C [3]. Another product of this reaction, 1,2-diacylglycerol (DG), activates Ca^{2+},phospholipid-dependent protein kinase C (C-kinase), which synergizes with Ca^{2+} to potentiate secretion not only in platelets but also in many cells [4,5]. Phorbol ester, which can substitute for DG to activate C-kinase [6], also activates platelets, but slowly and differently from agonists [7–9]. Yet some recent studies have demonstrated that pretreatment with phorbol esters for 2–3 min inhibits Ca^{2+} mobilization in several cell types [10–15]. In a sequential set of rapid responses, such activation of C-kinase is too slow to be significant. To see if activation of C-kinase could occur rapidly enough to modify agonist actions, we looked at early effects of phorbol esters.

Abbreviations: PMA, phorbol 12-myristate 13-acetate; DG, 1,2-diacylglycerol; 4α-PDD, 4α-phorbol didecanoate; C-kinase, Ca^{2+},phospholipid-dependent protein kinase; IP_3, *myo*-inositol 1,4,5-triphosphate; MLCK, myosin light chain kinase; PRP, platelet-rich plasma

2. EXPERIMENTAL

Shape change of washed platelets was monitored by absorbance (A) scans as described in the legend to fig.1. As shown (left panel), 0.025 U/ml thrombin induced shape change in 15 s as indicated by a rapid rise in A and loss of the oscillatory response characteristic of stirred discoid platelets (monitored by phase microscopy). PMA (Sigma, St. Louis) added 10 s before thrombin inhibited these responses: a strong effect was seen at 0.5 nM PMA, while 100 nM PMA prevented the rise in A completely. The scanning electron micrographs of fig.2 show that platelets pretreated with PMA were

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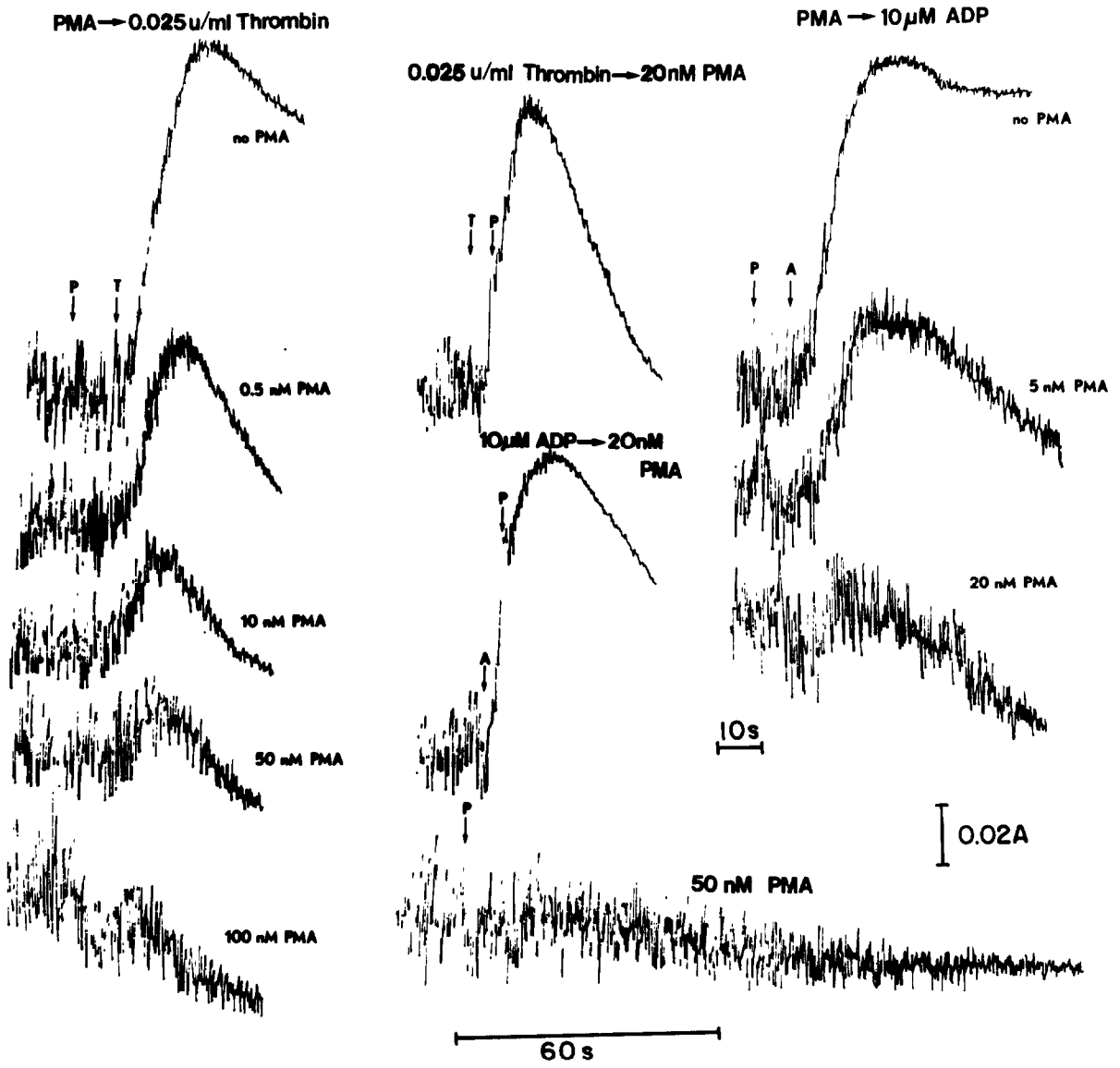

Fig.1. The effect of PMA on shape change induced by thrombin or ADP. Blood was drawn from healthy volunteers as approved by the Human Subjects Committee. Platelets were washed twice as described [27] and suspended in a Tyrode-Hepes buffer (TH buffer) containing 140 mM NaCl, 2.5 mM KCl, 0.5 mM NaH_2PO_4, 10 mM $NaHCO_3$, 0.1 mM $MgCl_2$, 10 mM Hepes, 0.35% bovine serum albumin and 0.1% glucose. During the washing 0.1 mg/ml apyrase (Sigma A-6132, 1.2 ADP U/mg) was added. Absorbance (A) of stirred platelets was monitored in a Gilford spectrophotometer at 37°C according to Born [28] using maximum sensitivity. EGTA was added at 1 mM 30 s before each incubation. The traces are typical of several similar experiments using platelets from different donors.

inhibited from extending filopodia and undergoing shape change in response to thrombin. PMA at lower concentrations similarly inhibited ADP-induced shape change (fig.1, right). PMA added 5 s after thrombin or ADP did not restore A or the oscillatory signal (right center). PMA alone caused a slow loss of oscillatory record after about 2 min without increase in A (bottom right); irregular

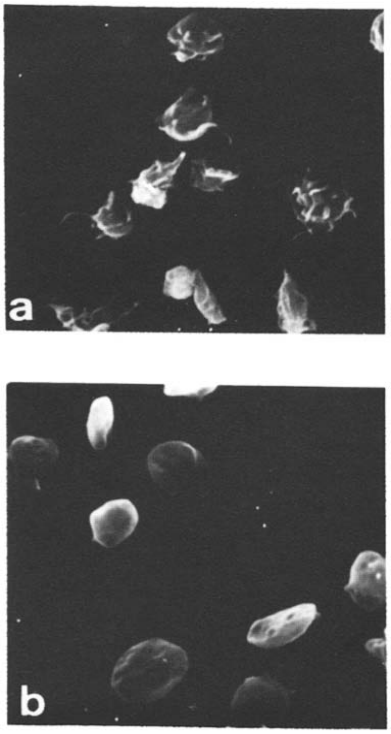

Fig.2. Scanning electron micrographs of platelets treated with ADP or with PMA prior to ADP. Washed platelets were treated with solvent (a) or with 20 nM PMA for 10 s (b) prior to 10 μM ADP for 15 s (both) and then fixed with 2% formaldehyde, 0.2% glutaraldehyde in Tyrode buffer for 30 min then allowed to settle on polylysine-L-coated coverslips for 30 min. The coverslips were rinsed with buffer and fixed overnight at 4°C in 2% glutaraldehyde in Tyrode buffer. The samples were dehydrated in an ethanol series, critical point dried and coated with gold platinum (approx. 150 Å). The pretreatment with PMA prevented the emergence of fine filopodia and the change in shape to irregular spheres.

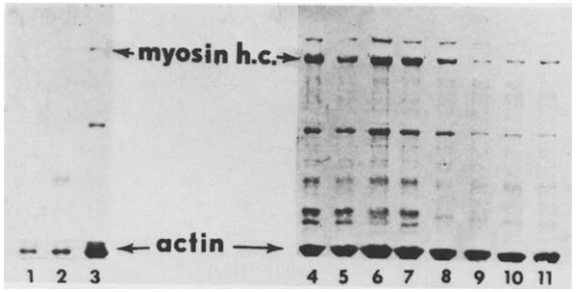

Fig.3. SDS gel electrophoresis of platelet cytoskeletons after stimulation with thrombin, ADP, or PMA alone or in conjunction with agonists. Cytoskeletons were prepared by adding an equal volume of 2% Triton X-100, 20 mM imidazole-HCl, 20 mM EGTA, pH 7.0, to washed platelet suspensions, left on ice for 5–15 min before centrifugation at 1400 × g for 10 min, and washed with a 1:1 mixture of TH buffer and Triton lysis buffer. The precipitates were dissolved in Laemmli buffer and electrophoresed on a 7.5% SDS-containing gel by the method of Laemmli [29]. Experimental conditions: 0.5 ml platelets (1 × PRP concentration) in Tyrode-Hepes buffer containing 1 mM EGTA were either treated with 50 nM PMA alone (lanes 2,3) or with agonists (lanes 5,6,9,10). Lanes: 1, control; 2, PMA 30 s; 3, PMA 2 min; 4, 0.025 U/ml thrombin 10 s; 5, PMA followed 10 s later by thrombin 10 s before lysis; 6, 0.025 U/ml thrombin followed 10 s later by PMA for 50 s before lysis; 7, 0.025 U/ml thrombin for 60 s; 8, ADP 10 s; 9, PMA followed 10 s later by ADP 10 s; 10, ADP followed 10 s later by PMA 50 s; 11, ADP 60 s. The three polypeptides which appear above actin in cytoskeletons after thrombin (lanes 4–7) are due to fibrin [30].

spheres were observed as previously reported using electron microscopy [7].

Shape change is believed to be caused at least in part by the interaction of actin with myosin activated by the phosphorylation of the myosin regulatory light chain by Ca^{2+},calmodulin-dependent light chain kinase (MLCK) [1], which is correlated with increased association of myosin with the cytoskeleton [16,17]. PMA treatments altered the composition of cytoskeletons by SDS gel electrophoresis (fig.3). After addition of thrombin or ADP, myosin and actin increased in the cytoskeletons (lanes 4,7,8,11) as reported for thrombin but not previously observed for ADP [16]. Pretreatment with 50 nM PMA for 10 s inhibited the association of myosin with cytoskeletons induced by 0.025 U/ml thrombin (cf. lanes 4,5) or by 10 μM ADP (lanes 8,9) but had little or no effect when added 5 s after the agonists (cf. lanes 6,7 and 10,11). Cytoskeletons of platelets treated with PMA (50 nM) for 2 min but not for 30 s contained increased amounts of actin and a small amount of myosin (lanes 2,3). Increased myosin and actin in cytoskeletons parallels the occurrence of shape change as manifested by the loss in oscillatory signals in fig.1, but maximum shape change occurs after ADP with less cytoskeletal myosin than is observed after thrombin.

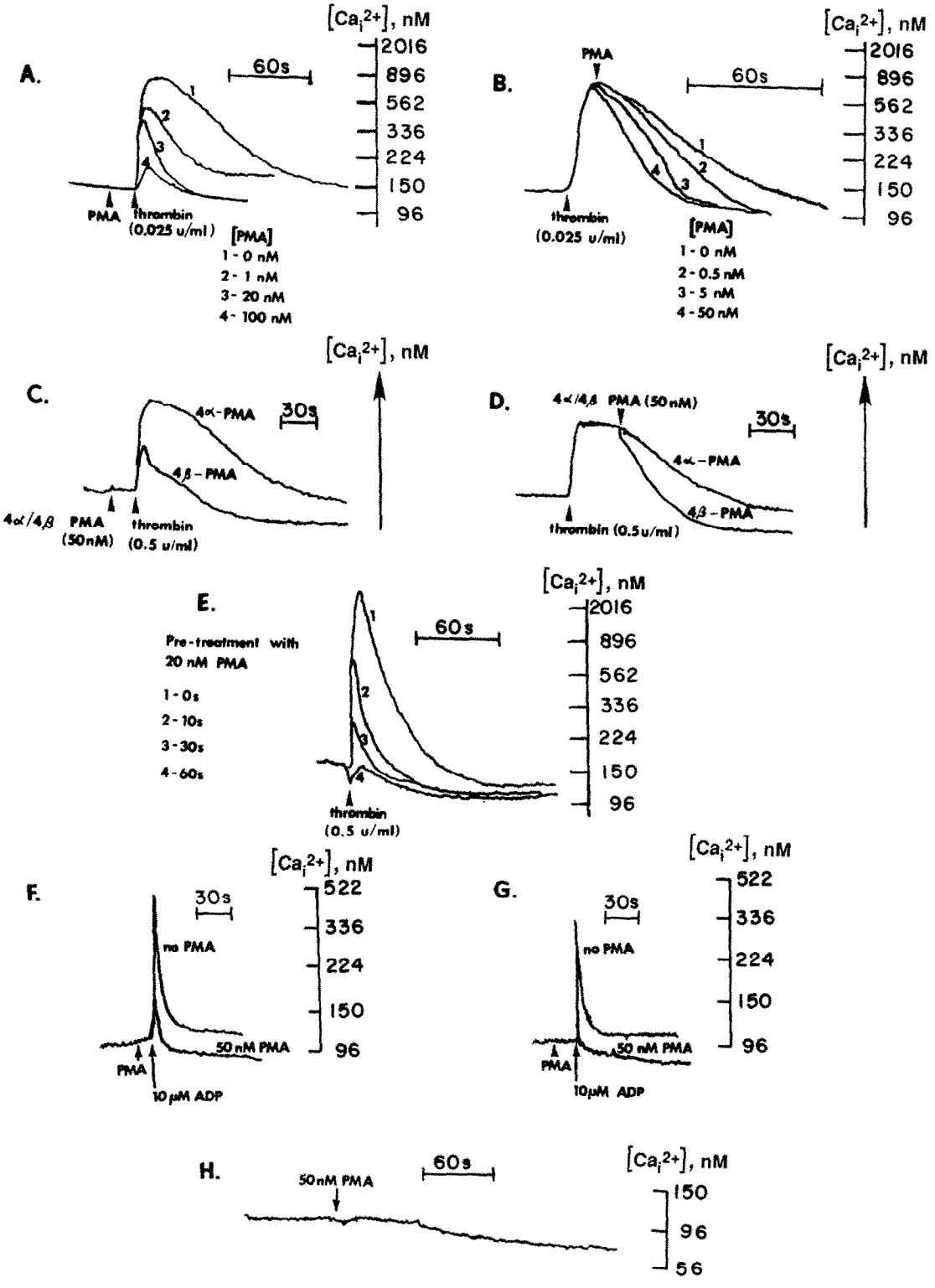
A.
[Ca$_i^{2+}$], nM
2016
896
562
336
224
150
96
60s
1
2
3
4
PMA
thrombin (0.025 u/ml)
[PMA]
1 - 0 nM
2 - 1 nM
3 - 20 nM
4 - 100 nM
B.
PMA
60s
thrombin (0.025 u/ml)
[PMA]
1 - 0 nM
2 - 0.5 nM
3 - 5 nM
4 - 50 nM
C.
4α-PMA
4β-PMA
30s
4α/4β PMA (50nM)
thrombin (0.5 u/ml)
D.
4α/4β PMA (50 nM)
30s
4α-PMA
4β-PMA
thrombin (0.5 u/ml)
E.
Pre-treatment with 20 nM PMA
1 - 0s
2 - 10s
3 - 30s
4 - 60s
60s
thrombin (0.5 u/ml)
F.
30s
no PMA
50 nM PMA
PMA
10 μM ADP
522
336
224
150
96
G.
30s
no PMA
50 nM PMA
PMA
10μM ADP
H.
50nM PMA
60s
150
96
56

Fig.4. Effects of PMA on calcium transients induced by thrombin or ADP. Platelets suspended in Mill's buffer [27] were incubated with fura-2 (Molecular Probes, Junction City, OR) at 2 μg/ml for 30 min at 37°C in the dark. After being sedimented and resuspended, the loaded platelets were rested for 15–30 min at 37°C. Ca^{2+} measurements were performed as in [18,19] in the presence of 1 mM EGTA (A,B,E,G,H) or 1 mM $CaCl_2$ (C,D,F).

We measured Ca_i^{2+} using fura-2 [18] as described [19]. Fig.4 shows the rapid rise in Ca_i^{2+} after thrombin or ADP. The signal after thrombin lasted about 120 s, while that after ADP only about 20 s. We observed a remarkably rapid dose-dependent inhibition of the calcium signal by PMA added 10 s before agonists. The correlation of PMA inhibition on the Ca^{2+} signals and the shape change induced by 0.025 U/ml thrombin was as follows: PMA at 1, 10, and 50 nM inhibited shape change (10 s) by 50, 70, and 90%, and suppressed Ca^{2+} signals (as measured by the area under the curves) by 50, 80, and 85% in the absence of external Ca^{2+}. The transient, smaller Ca^{2+} signal produced by 10 μM ADP in the absence of external Ca^{2+} was completely inhibited by pretreatment with 20 nM PMA for 10 s, correlating with the greater sensitivity of ADP-induced shape change to PMA. We also observed inhibition of 80% of the Ca^{2+} signal induced by 0.5 U/ml thrombin with 50 nM PMA even with external Ca^{2+} (fig.4C). Remarkably, PMA added after thrombin increased the rate of return of the calcium signal to the baseline and even caused an overshoot (fig.4B,D). This result may be due to the use of the fura-2 method which has higher sensitivity than the quin-2 method, particularly in measuring decreases in Ca^{2+} [19]. 4α-PDD, which cannot activate C-kinase, did not produce inhibition (fig.4C,D).

3. DISCUSSION

PMA acts more rapidly than previously observed in platelet studies: e.g. 10–20 ng/ml PMA was used for 3 min to produce a 90% inhibition of the thrombin-induced Ca^{2+} signal [10], or 160 nM PMA was used for 2 min [11,12]. Fig.4E shows that the inhibition of calcium signal by 20 nM PMA is essentially complete after less than 60 s. In previous studies, platelets would have already undergone atypical, PMA-induced shape change before the addition of agonists. These effects of PMA appear to be due to C-kinase activation because they were mimicked by 1-oleoyl-2-acetylglycerol, an analogue of DG, but not by 4α-PDD, which cannot activate C-kinase, and they occurred as rapidly as the phosphorylation of 47 kDa protein, which reached 70% of maximum by 10 s [6]. Does this mean that the normal decline in the calcium signal is due to some C-kinase activation? Or, do mechanisms such as those dependent on cyclic AMP work to remove the calcium signal soon after it occurs? It has recently been reported that phorbol ester can prevent the agonist-dependent inhibition of platelet adenylate cyclase which takes place via an inhibitory N_i protein [20]. On the other hand, C-kinase phosphorylates phospholamban to activate the calcium pump of heart sarcoplasmic reticulum [21]. Recently, we found that a membrane fraction from PMA-treated platelets takes up labelled calcium at about 3-times the rate of control membranes (unpublished).

Pretreatment with PMA for more than 1 min has been reported to inhibit thrombin-induced Ca^{2+} transients by suppressing IP_3 production [10,12]. In addition, PMA phosphorylates an inhibitory site (C-kinase site) on the myosin P light chain, reducing the contractility of the actomyosin [25], an effect which could increase inhibition of myosin-dependent events, such as shape change, if it occurred rapidly.

Much evidence supports the idea of a synergism between Ca^{2+} and C-kinase in the mechanism of secretion (review [5]). However, the present data show that PMA, presumably by activating C-kinase can act to inhibit early Ca^{2+}-mediated events. We suggest that C-kinase may act as a temporal regulator for a series of sequential responses, turning off early events and stimulating those that occur later in a sequence.

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