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Comparison of the effects of phorbol 12-myristate 13-acetate and prostaglandin E_1 on calcium regulation in human platelets

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We compared the effects of phorbol 12-myristate 13-acetate (PMA) with those of prostaglandin E_1 (PGE_1) on the calcium transient in intact platelets and on $^{45}Ca^{2+}$ uptake in saponin-treated platelets and microsomal fractions to determine the roles of protein kinase C and cyclic AMP in calcium sequestration. In intact platelets, PMA, like PGE_1, stimulated the return of the calcium transient to resting values after a thrombin stimulus, but only the PGE_1 effect was reversed by adrenaline. Both PMA and PGE_1, when added before saponin, stimulated ATP-dependent $^{45}Ca^{2+}$ uptake into the permeabilized platelets. Thrombin also stimulated $^{45}Ca^{2+}$ uptake into saponin-treated platelets. Uptake of $^{45}Ca^{2+}$ was increased in microsomal preparations from platelets pretreated with PMA or PGE_1. PMA did not increase the cyclic AMP content of control or thrombin-treated platelets, and it induced a pattern of protein phosphorylation in ^{32}P-labelled platelets different from that with PGE_1. In correlation with the increased uptake of calcium in the saponin-treated preparation, we measured a rapid translocation of protein kinase C from supernatant to cell fraction after the addition of PMA. Our results suggest that activation of protein kinase C enhances calcium sequestration independently of an effect on cyclic AMP content in platelets. This activation could play a physiological role in the regulation of the calcium transient.

INTRODUCTION

Stimuli which increase cyclic AMP have been shown to stimulate calcium sequestration in microsomal fractions from human platelets (Käser-Glanzmann *et al.*, 1979; Fox *et al.*, 1979). More recently, not only cyclic AMP-dependent kinase and calmodulin-dependent kinase, but also Ca^{2+}-activated phospholipid-dependent protein kinase (protein kinase C; see Nishizuka, 1986), have been shown to stimulate both calcium uptake and sequestration and phosphorylation of a 22 kDa protein, phospholamban, in heart sarcoplasmic reticulum (Tada *et al.*, 1979; Movsesian *et al.*, 1984; Iwasa & Hosey, 1984; Kranias, 1985). We previously found that PMA, which directly stimulates protein kinase C (Castagna *et al.*, 1982), immediately accelerated the decline in the calcium transient of human platelets when added after low doses of thrombin, as detected by fura-2 (Yoshida *et al.*, 1986). This is similar to the effect of PGI_2 (Zavoico & Feinstein, 1984), which increases cyclic AMP in platelets. This similarity suggested that in platelets, as in cardiac muscle, activating protein kinase C might directly affect calcium sequestration.

This question cannot be resolved with intact platelets, since the regulatory mechanisms are complex. In human platelets as in many other cell types, agonist action results in the activation of phospholipase C which acts on phosphatidylinositol 4,5-bisphosphate to form *myo*-inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 releases Ca^{2+} from the dense tubular system, and diacylglycerol stimulates protein kinase C (Nishizuka, 1986). Protein kinase C regulates the amount of IP_3 both by inhibiting its formation (Watson & Lapetina, 1985; Rittenhouse & Sasson, 1985) and by stimulating its degradation (Connolly & Majerus, 1986; Molina y Vedia & Lapetina, 1986). Activation of protein kinase C could therefore decrease free calcium by decreasing IP_3 amounts and hence calcium release. In intact platelets, it could also increase the extrusion of calcium across the plasma membrane to the external solution, or finally it could cause increased sequestration into internal membrane compartments. We were stimulated to look for an effect on sequestration in platelets by the strong effect of protein kinase C on calcium uptake in cardiac sarcoplasmic reticulum (Movsesian *et al.*, 1984; Iwasa & Hosey, 1984).

Brass & Joseph (1985) showed that controlled use of saponin would permeabilize the platelet plasma membrane while preserving the activity of the intracellular calcium-accumulating compartment. A similar approach was used by Authi *et al.* (1986) to study IP_3-induced calcium release. We have taken advantage of this method to compare the effects of PMA and PGE_1 on calcium uptake into an intracellular membrane compartment in saponin-treated platelets, thus eliminating regulatory pathways involving the plasma membrane.

EXPERIMENTAL

Preparation of platelet suspensions

Blood was donated by healthy volunteers under informed consent, who denied recent medication. After the first few ml were discarded, venous blood from the

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; PG, prostaglandin; IP_3, inositol trisphosphate.

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antecubital vein was allowed to flow freely into $\frac{1}{10}$ vol. of buffer containing 85 mM-sodium citrate and 65 mM-citric acid. Platelets were isolated from platelet-rich plasma, after adjusting the pH to 6.5, by centrifugation at 650 *g* for 10 min. They were suspended in a buffer containing 140 mM-NaCl, 2.7 mM-KCl, 5.5 mM-glucose and 0.1 mg of apyrase (Sigma A-6132; 1.2 ADP unit/mg)/ml and 10 mM-Pipes buffer, pH 6.5, at 1.5×10^8–3×10^8 platelets/ml. For the measurement of calcium transients, the platelet suspension was incubated with 2 μg of fura-2 (Molecular Probes, Junction City, OR, U.S.A.)/ml, diluted from a 2 mg/ml stock solution in dimethyl sulphoxide, for 30–60 min at 37 °C in the dark. For phosphorylation experiments, platelets were suspended in the Pipes buffer to $\frac{1}{5}$ vol. of platelet-rich plasma and incubated with 1 mCi of $[^{32}P]P_i$ (ICN Radiochemicals, Irvine, CA, U.S.A.)/ml for 1 h at 37 °C. After collection by centrifugation at 200 *g* for 5 min, the platelets were suspended in a Tyrode–Hepes buffer containing 140 mM-NaCl, 2.7 mM-KCl, 12 mM-$NaHCO_3$, 5 mM-Hepes, 0.42 mM-NaH_2PO_4, 1 mM-$MgCl_2$, plus 5.5 mM-glucose and 0.1 mg of apyrase/ml at pH 7.4. In experiments with saponin-treated platelets, the platelets were suspended in high-KCl buffer in which NaCl was replaced by KCl, the Mg^{2+} was increased to 5 mM and apyrase was replaced with 5 mM-phosphocreatine and 10 units of creatine kinase/ml (both from Sigma Chemical Co.); pH was 7.1. The cell count was adjusted to 3.5×10^8/ml.

Measurement of cytosolic free calcium

The concentration of cytoplasmic free Ca^{2+} was measured by using fura-2 as described by Grynkiewicz *et al.* (1985) and Pollock *et al.* (1986). Measurement of fluorescence emission at 510 nm after excitation at 340 nm in some experiments, and in others with dual wavelength excitation, at both 340 and 380 nm, was carried out in spectrofluorimeters of special design (Johnson Foundation, University of Pennsylvania) at 37 °C with stirring. We thank Dr. Barbara Corkey, Clay Glennon and Dr. Bernard J. Himpens for helping us in obtaining the measurements. Free Ca^{2+} was estimated by using a Ca^{2+}–dye dissociation constant of 224 nM as described by Pollock *et al.* (1986). Fluorescence from external dye, assessed by the addition of excess EGTA, was subtracted from the total fluorescence signal. As the platelets slowly leak fura-2, it was necessary to work quickly, and the duration of each experiment was 60–90 min. Minimum fluorescence was determined by adding digitonin to 50 μM with 1 mM-EGTA and 20 mM-Tris/HCl, pH 8.5. The maximum fluorescence was determined by further addition of 10 mM-$CaCl_2$. The inactive phorbol ester 4α-phorbol didecanoate and PMA were both used in these experiments diluted from stocks in dimethyl sulphoxide.

$^{45}Ca^{2+}$ uptake in saponin-treated platelets

These experiments were performed as described by Brass & Joseph (1985), with slight modifications. Platelets were suspended in high-KCl buffer at an initial dilution of 3.5×10^8/ml to prevent aggregation. A Ca^{2+}–EGTA buffer (1 mM-EGTA/0.746 mM-$CaCl_2$) was used to control the free calcium concentration at 0.5 μM, calculated by the program of Chantler *et al.* (1981), which includes the effects of pH and Mg^{2+} ions. Ruthenium Red (2 μM) and oligomycin (5 μg/ml) were used as mitochondrial inhibitors. After incubation with PMA, PGE_1 or thrombin for 2 min at 37 °C, 5–10 μCi of $^{45}Ca^{2+}$/ml and 15–20 μg of saponin/ml were added in rapid sequence to initiate uptake. At the intervals indicated, 200 μl portions of platelet suspension were removed and filtered through 0.45 μm-pore-size micro-filtration membranes (Nucleopore Co., Pleasanton, CA, U.S.A.) on a vacuum-filtration manifold (Millipore Co., Bedford, MA, U.S.A.). The filters, pre-soaked in the high-KCl buffer containing 0.5 mM-EGTA, were washed with 2×3 ml of the same buffer. $^{45}Ca^{2+}$ retained by the filters was measured in a Beckman LS 3155T scintillation spectrometer after adding 5 ml of scintillant (ACS II; Amersham Co., Arlington Heights, IL, U.S.A.).

Preparation of the microsomal fraction

This was done as follows: platelets (10 × platelet-rich-plasma concentration) were suspended in a buffer containing 30 mM-KCl, 5 mM-$MgCl_2$, 20 mM-Tris, 2 mM-ATP, 0.3 M-sucrose and 5 mM-EGTA, pH 7.0, and were treated with 20 nM-PMA or 1 μM-PGE_1 for 5 min at room temperature, then sonicated with a Branson instrument for 4×15 s on ice. Leupeptin (0.2 mM), phenylmethanesulphonyl fluoride (0.5 mM), benzamidine (1 mM) and 5 mM-EGTA were added immediately before sonication. The sonicated preparations were centrifuged at 20000 *g* for 20 min at 4 °C and the supernatants re-centrifuged at 100000 g_{av} for 1 h at 4 °C to obtain a microsomal fraction (microsomes). Uptake of $^{45}Ca^{2+}$ was measured in 0.5 ml portions in a buffer containing 120 mM-KCl, 10 mM-Hepes, pH 7.1, 5 mM-$MgCl_2$, 5 mM-ATP, 50 mM-NaH_2PO_4 with 20–100 μg of added microsomes at 37 °C. At various time intervals, 500 μl samples were filtered, washed and counted for radioactivity as described above.

Assay of cyclic AMP

After incubation of platelet suspensions (0.5 ml) with the appropriate agonists, the reactions were terminated by adding 0.5 ml of 12% (v/v) trichloroacetic acid. The samples were left for 10 min on ice, and then centrifuged at 10000 *g* for 10 min at 4 °C to obtain the supernatant. The trichloroacetic acid was extracted with 3×10 ml of diethyl ether, and the residual ether was evaporated under a stream of nitrogen while warmed at 50–70 °C. The cyclic AMP in these samples was determined by radioimmunoassay (RPA.508 kit) from Amersham.

Phosphorylation studies

Platelets labelled with ^{32}P were incubated with the appropriate reagents, and phosphate incorporation was stopped with $\frac{1}{5}$ vol. of 5-fold-concentrated Laemmli (1970) buffer, followed by boiling for 2–5 min. Equal portions of samples were electrophoresed on 13%-acrylamide gels in the presence of SDS (Laemmli, 1970). Gels were stained with Coomassie Blue and dried between dialysis membranes, and exposed to X-Omat AR film (Eastman–Kodak, Rochester, NY, U.S.A.) at −70 °C with Dupont Cronex lightning screens.

Translocation and assay of protein kinase C in saponin-treated platelets

Platelet suspensions for protein kinase C activity were prepared as described for the saponin-treated platelets, with the following modifications: apyrase was omitted and 2 mM-EGTA was added to the Pipes buffer; phosphocreatine and creatine kinase were omitted from,

and 5 mM-EGTA and 100 μg of leupeptin/ml were added to, the high-KCl buffer. Samples of the suspension (750 μl of 3.5×10^8 cells/ml) were added to plastic tubes at 37 °C. Various concentrations of PMA or dimethyl sulphoxide as a solvent control were added for 10 s before the addition of 37.5 μg of saponin (50 μg/ml final concn.). The volume of the PMA or dimethyl sulphoxide was less than 1 % of the platelet suspension. The platelets were incubated at 37 °C for 15 min after addition of saponin, and were then spun down at 11000 *g* for 30 s. The supernatant fraction was carefully removed by pipette, and glycerol was added to a final concentration of 25 % (v/v). The cell pellet was resuspended in 200 μl of ice-cold high-KCl buffer containing 0.1 % Triton X-100 and 25 % (v/v) glycerol. The samples were frozen and stored at −20 °C until assayed for protein concentration and kinase activity. Freezing the samples for several days under these conditions had no effect on kinase activity.

Kinase activity was measured by the procedure of Castagna *et al.* (1982), with the following modifications. The reaction mixture (100 μl) contained 2.5 nmol of [γ-^{32}P]ATP (ICN Radiochemicals) at 2.2×10^6 c.p.m./nmol. Phorbol ester was added to a concentration of 100 nM, $CaCl_2$ to a final concentration of 10 μM and phosphatidylserine to a final concentration of 20 μg/ml. In controls, 5 mM-EGTA was substituted for PMA, $CaCl_2$ and phosphatidylserine. To each 100 μl of reaction mixture, 25 μl of sample was added and then incubated at 37 °C for 5 min. The reaction was stopped with an equal volume of ice-cold 25 % trichloroacetic acid containing 4 % (w/v) $Na_4P_2O_7$, and bovine serum albumin (100 μg) was added. The mixture was then filtered on a Millipore filtration manifold through a glass-fibre filter presoaked in 5 % trichloroacetic acid containing 2 % $Na_4P_2O_7$, washed extensively, and the ^{32}P retained was counted as described above after subtraction of non-specific binding.

Other methods and reagents

PMA and the 4α-didecanoate analogue (from Sigma Chemical Co.) were kept frozen as 400 μM stock solutions in dimethyl sulphoxide and diluted immediately before use. Dimethyl sulphoxide was added to control samples (0.2 % in most experiments, and 1 % in the translocation studies). Lactate dehydrogenase activity was measured with Sigma kit 340-LD. All other reagents were of analytical grade.

RESULTS

Fig. 1 shows typical Ca^{2+} transients as monitored by fura-2 after addition of 0.1 unit of thrombin/ml (upper curves). The addition of PMA immediately accelerates the rate at which the fura signal returns to the baseline when it is added at the peak of the Ca^{2+} transient (Fig. 1, left, lower tracing). The inactive analogue 4α-phorbol didecanoate did not produce any change in the thrombin transient (results not shown). PGE_1, like PMA, also increases the rate of return of Ca^{2+} after thrombin (right, lower tracing) as reported with PGI_2 with quin-2 as an indicator (Zavoico & Feinstein, 1984). These investigators showed that adrenaline, which inhibits adenylate cyclase activity, reversed the effect of PGI_2. We confirmed this for PGE_1, and found that, by contrast, adrenaline does not reverse the effect of PMA (Fig. 1, middle tracings).

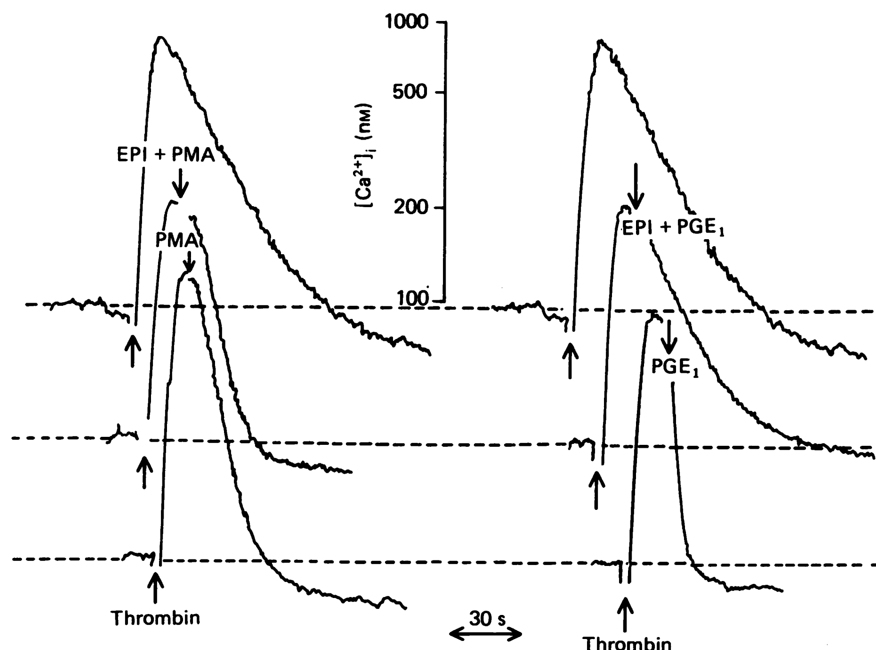

Fig. 1. Effect of PMA and PGE_1 on the Ca^{2+} transients induced by thrombin as monitored by fura-2

Thrombin (0.1 unit/ml) was added to fura-2-loaded platelets in the presence of 1 mM-EGTA. The control recording is shown in the upper set of curves. Next, either 20 nM-PMA or 1 μM-PGE_1 was added at the peak of the Ca^{2+} transient (lower recordings). Both accelerated the rate of return of fluorescence to the baseline. Adrenaline (EPI; 20 μM) added shortly before PGE_1 inhibited the effect of PGE_1, but did not alter the effect of PMA (compare the two middle curves). We show a tracing typical of three similar experiments. The calcium concentration in the platelets, $[Ca^{2+}]_i$, was measured as described above.

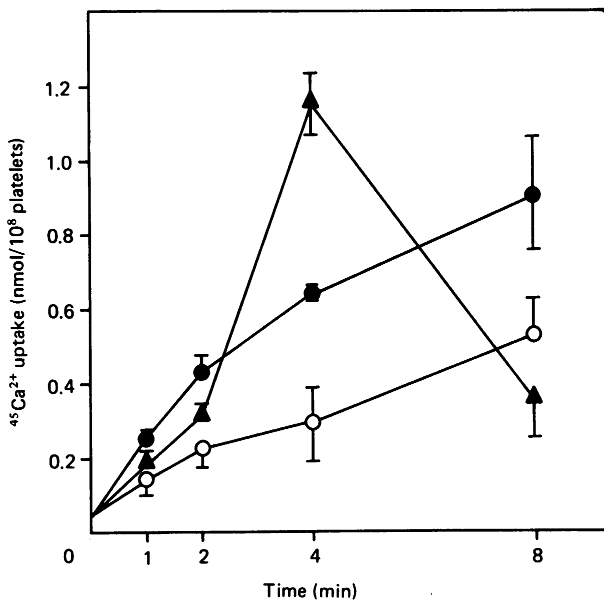

Fig. 2. Effect of PMA or PGE_1 on $^{45}Ca^{2+}$ uptake in saponin-treated platelets

Platelets in high-KCl buffer were treated with 20 nM-PMA (●) or 1 μM-PGE_1 (▲) or with 0.2% dimethyl sulphoxide (○) for 2 min. Then saponin and $^{45}Ca^{2+}$ were added in rapid sequence. At intervals, samples were filtered, washed and counted for radioactivity to measure the amount of $^{45}Ca^{2+}$ associated with the particulate fraction. The uptake was dependent on ATP. The values shown are means of two typical experiments.

To exclude effects of PMA or PGE_1 on increased calcium extrusion, we examined their effects on saponin-treated platelets. Using concentrations of saponin of 15–25 μg/ml per 3.5×10^8 platelets, similar to that used by Brass & Joseph (1985), we permeabilized the plasma membrane while maintaining the integrity of internal membrane systems that accumulate calcium. Fig. 2 shows a typical time course of $^{45}Ca^{2+}$ uptake in saponin-treated platelets in the presence of an ATP-regenerating system, as described above, with free Ca^{2+} at 0.5 μM (Ca^{2+}–EGTA buffer) in the presence of mitochondrial inhibitors. Pretreatment with PMA 2 min before saponin treatment increases the rate and extent of $^{45}Ca^{2+}$ uptake nearly 2-fold, as shown. The maximum effect is observed at 10 nM-PMA. A rapid effect of PMA at 10 nM is observed even when added immediately before saponin. PMA added 2 min after saponin was ineffective. Preincubation with PGE_1 also stimulates $^{45}Ca^{2+}$ uptake as shown, but it returns to control values by 8 min. Most of the $^{45}Ca^{2+}$ that is taken up is released by Ca^{2+} ionophore. Similar results are observed when we prepared microsomes from platelets pretreated with PMA or PGE_1. As shown in Fig. 3, both PMA and PGE_1 potentiate $^{45}Ca^{2+}$ uptake into the microsomal fraction.

As shown in Table 1, when we added PMA to the microsomes from non-treated platelets there was no increase in $^{45}Ca^{2+}$ uptake, whereas preincubation with PMA resulted in nearly a 2-fold increase in Ca^{2+} uptake. Furthermore, PMA increases $^{45}Ca^{2+}$ uptake in microsomes prepared from PMA-treated platelets by another 20%. Table 2 shows the effects of PMA and thrombin on

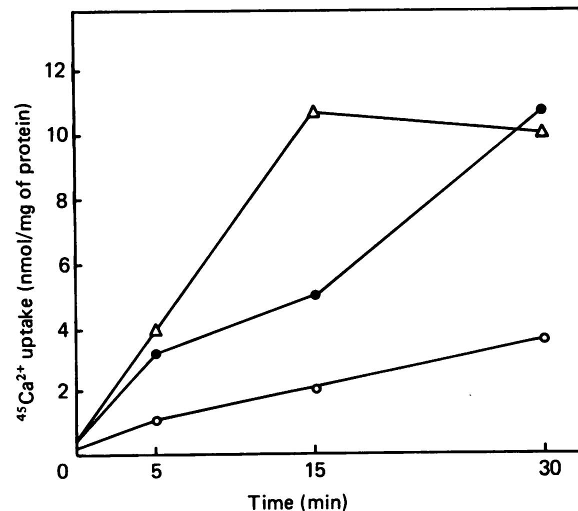

Fig. 3. Effect of pretreatment with PMA or PGE_1 on $^{45}Ca^{2+}$ uptake into microsomes

Platelets were treated with 20 nM-PMA (●) or 1 μM-PGE_1 (△) or dimethyl sulphoxide (○) for 5 min, then sonicated. The sonicated material was first centrifuged at 19000 g for 20 min, and the supernatant was centrifuged at 90000 g for 1 h to obtain a microsome fraction. The uptake of $^{45}Ca^{2+}$ was measured as described in the Experimental section. The time course shown is typical of seven similar experiments.

net calcium uptake into intact and saponin-treated platelets. Pretreatment of saponin-treated platelets with thrombin (0.1 unit/ml) for 2 min increases $^{45}Ca^{2+}$ uptake to 150% of the control value, more than when it is added to intact platelets, whereas 20 nM-PMA increases $^{45}Ca^{2+}$ uptake into saponin-treated platelets 2-fold and slightly decreases uptake in intact platelets. PMA inhibits the stimulation of net Ca^{2+} influx by thrombin in intact platelets.

Tapley & Murray (1985) showed that PMA induced the translocation of protein kinase C from the soluble to a crude particulate fraction in platelets. We observed such translocation using a microsomal preparation (Table 3). Using the saponin-treated preparation, we obtained a rapid fractionation of the platelet into soluble and insoluble compartments for the study of trans-

Table 1. Effects of PMA and thrombin on $^{45}Ca^{2+}$ uptake into the microsomal fraction

Platelets were treated with 20 nM-PMA or dimethyl sulphoxide (control) for 5 min. The microsomal fraction was prepared as described in the Experimental section, and the uptake was measured without or with added PMA (50 nM). The results are expressed as means ± S.E.M. for seven identical experiments. *Significant ($P < 0.05$) compared with PMA-pretreated alone.

	$^{45}Ca^{2+}$ uptake (% of control)
Control + PMA added	95.6 ± 7.0
PMA-pretreated	172 ± 19.5
PMA-pretreated + PMA added	212 ± 25.2*

Table 2. Effects of PMA and thrombin on $^{45}Ca^{2+}$ uptake into intact and saponin-treated platelets

Platelets were treated with 20 nM-PMA or 0.1 unit of thrombin/ml for 2 min, alone or together, then $^{45}Ca^{2+}$ was added with or without saponin. Uptake was allowed to proceed for 2 min. Results are means ± S.E.M. for two representative experiments in triplicate. *Significant ($P < 0.05$) compared with the appropriate control.

	$^{45}Ca^{2+}$ uptake (% of control)
Control + saponin	100 ± 8.6
Control, no saponin	38.2 ± 8.4
Thrombin + saponin	150 ± 18.2*
Thrombin, no saponin	131 ± 4.0*
PMA + saponin	207 ± 28.6
PMA, no saponin	89.1 ± 6.9*
PMA + thrombin + saponin	175 ± 12.6*
PMA + thrombin, no saponin	74.9 ± 0.7*

location. Measurement of lactate dehydrogenase showed that 85% or more of this soluble protein is present in the supernatant after treatment with 50 μg of saponin/ml for 15 min. In control platelets, 79 ± 6% of the protein kinase C is in the supernatant. With increasing doses of PMA, activity is lost from the supernatant and appears in the pellet, as shown in Fig. 4. At 10 nM-PMA there is nearly a doubling of the activity in the pellet fraction. This concentration gives maximum uptake of $^{45}Ca^{2+}$ in saponin-treated platelets which are pretreated for a longer time.

One critical question in this study is whether PMA might stimulate Ca^{2+} sequestration by changing cyclic AMP concentrations. This is a possibility, since it has been reported that activation of protein kinase C can reverse the inhibition of adenylate cyclase caused by agonists (Katada *et al.*, 1985). Table 4 shows that PMA by itself or in combination with thrombin does not

Table 3. Translocation of protein kinase C to platelet microsomes

Microsomes were prepared as described after exposure to 100 nM-PMA or an equal volume of dimethyl sulphoxide as a control. Kinase activity was measured by the method of Tapley & Murray (1985), with 2 mM-EGTA as a control. Total activity is the activity of the microsomal and the 100000 *g* supernatant fractions, and the activities of the fractions are compared with the corresponding total activities. Activity in EGTA was 5–12% of total activity in any given preparation. The results are means ± S.E.M. for four experiments, three with units of platelets obtained from Red Cross and one experiment with freshly donated platelets.

Pretreatment	Fraction	Activity (% of total)
PMA	Microsomes	83.4 ± 7.7
	Supernatant	15.5 ± 7.7
Control	Microsomes	17.6 ± 4.8
	Supernatant	84.2 ± 5.1

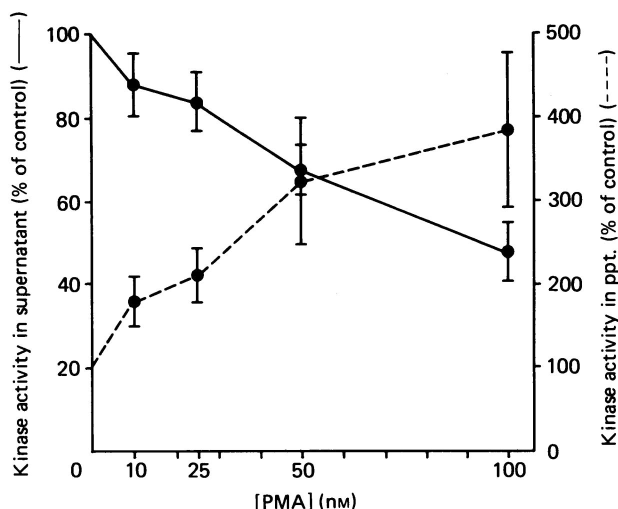

Fig. 4. Translocation of C-kinase in saponin-treated platelets from supernatant to pellet fractions with increasing concentrations of PMA

A 10 s incubation with PMA at the indicated concentrations was immediately followed by saponization for 15 min. Pellets were separated from supernatants by centrifugation and samples were mixed in 25% glycerol as described in the Experimental section. PMA-treated samples are compared with untreated controls that were saponin-treated under identical conditions. The continuous line represents the comparison between PMA-treated and untreated control supernatants. The broken line represents the comparison between PMA-treated and untreated control pellets. The results shown are of an experiment typical of three.

increase cyclic AMP over control values, but the cyclic AMP content is increased 6-fold after PGE_1 treatment. Thrombin effectively inhibits the increase in cyclic AMP produced by PGE_1, as shown.

We examined protein phosphorylation to see if there might be a common substrate for PMA and PGE_1 (Fig. 5). PGE_1 is reported to stimulate the phosphorylation of a 22 kDa protein, which may stimulate Ca^{2+} sequestration (Käser-Glanzmann *et al.*, 1979; Fox *et al.*, 1979).

Table 4. Cyclic AMP content under different conditions

Platelets were treated with the agents shown for 1 min. When PMA, PGE_1 or adrenaline was added with thrombin, they were added 10 s after thrombin to compare with Fig. 1. Results are means ± S.E.M. for three experiments in triplicate.

Treatment	Cyclic AMP (pmol/10^9 platelets)
Control	21.6 ± 0.4
+ PMA (20 nM)	18.0 ± 1.9
+ PGE_1 (1 μM)	150.7 ± 7.7
+ Thrombin (0.1 unit/ml)	18.1 ± 1.3
+ Adrenaline (10 μM)	16.3 ± 0.5
Thrombin + PMA	19.1 ± 0.0
Thrombin + PGE_1	28.5 ± 3.3
Thrombin + adrenaline + PMA	16.7 ± 1.0
Thrombin + adrenaline + PGE_1	25.2 ± 0.7

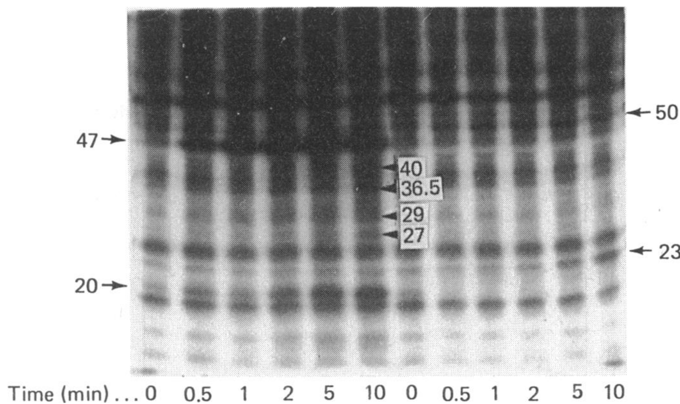

Fig. 5. Effect of PMA and PGE_1 on protein phosphorylation

Platelets which had been labelled with ^{32}P were treated with either 20 nM-PMA or 1 μM-PGE_1. At the time intervals indicated, samples were boiled with Laemmli (1970) buffer, and electrophoresed on a 13%-polyacrylamide gel. The M_r values ($\times 10^{-3}$) are from standards. The arrows point to certain strongly phosphorylated bands: note especially the 27 kDa band after PMA treatment (lane 6) and the 23 kDa band after PGE_1 treatment (lane 12).

We confirmed this finding (the molecular mass of the protein was calculated to be 23 kDa in our gel system). The phosphorylation as detected by autoradiography was marked by 2 min after PGE_1 addition (Fig. 5, lanes 7–12). On the other hand, PMA addition does not stimulate the phosphorylation of 23 kDa protein, but it potentiates the phosphorylation of proteins with major bands of 27 kDa, 29 kDa and 36.5 kDa, and in the regions of 40 kDa, in addition to the well-known substrates of protein kinase C, the 47 kDa and the 20 kDa band, known to be IP_3 5′-phosphomonoesterase (Connolly *et al.*, 1986) and myosin light chain (Naka *et al.*, 1983) respectively. Adrenaline inhibits the phosphorylation of the 23 kDa protein by PGE_1, which is expected if the phosphorylation of 23 kDa protein is cyclic-AMP-dependent (results not shown).

DISCUSSION

In this study we show that PMA increases the rate of return of the calcium transient when added after thrombin in intact platelets (Fig. 1) and enhances ATP-dependent $^{45}Ca^{2+}$ uptake in saponin-treated platelets (Fig. 2) and in microsomes (Fig. 3). These effects appear to be due to protein kinase C activation, because PMA directly stimulates protein kinase C (Castagna *et al.*, 1982), and an inactive phorbol, 4 α-phorbol didecanoate, could not substitute for PMA.

The stimulation of calcium uptake into microsomes from PMA-treated platelets is most likely into membranes from an intracellular compartment, since Menashi *et al.* (1984) reported that, with purified with purified membrane fractions, the surface membrane fraction did not sequester calcium in the presence of ATP. Pollock *et al.* (1987) reported that thrombin or PMA added shortly after ionomycin could accelerate the recovery of resting concentrations of $Ca^{2+}{}_i$ as detected by fura-2. They attributed the effect to the stimulation of increased Ca^{2+} efflux via the plasma membrane, because the continued presence of ionomycin is expected to 'short-circuit' sequestering organelles (Pollock *et al.*, 1986). However, this could not explain the effects on saponin-treated platelets or on the microsomes. On the other hand, PMA inhibits the stimulation by thrombin of $^{45}Ca^{2+}$ uptake into or binding by intact platelets (Table 2).

Although $^{45}Ca^{2+}$ uptake into microsomes increases when the platelets are pretreated with PMA, it is not increased when PMA is added directly to microsomes (Table 1). But PMA added to microsomes from PMA-treated platelets did increase $^{45}Ca^{2+}$ uptake. Adunyah & Dean (1986) also found that direct addition of PMA to microsomes did not increase $^{45}Ca^{2+}$ uptake into the purified microsome fraction; in fact they observed an inhibition. Since we observed that PMA treatment of intact platelets is followed by translocation of protein kinase C to the microsomes (Fig. 4), the absence of stimulation by direct addition of PMA may be due to the absence of protein kinase C from the microsomes. However, we were not able to cause increased calcium sequestration by direct addition of the kinase to the microsome fraction, with either partially or highly purified protein kinase C (gift from Dr. L. Diamond and Dr. Diane Husch, University of Pennsylvania). LePeuch *et al.* (1983) obtained similar results. These results suggest that a prior reaction must take place, involving the interaction of either PMA or protein kinase C with the membranes in order to observe enhanced uptake.

The results obtained with PMA are quite different from those seen after increased cyclic AMP. Käser-Glanzmann *et al.* (1979) and Fox *et al.* (1979) showed, respectively, that addition of cyclic AMP-dependent kinase or the pre-treatment of platelets with PGE_1 increased $^{45}Ca^{2+}$ uptake into microsomes and the phosphorylation of a 22 kDa protein. Although in cardiac tissue protein kinase C and cyclic AMP-dependent kinase increase the phosphorylation of the 22 kDa protein phospholamban, present in sarcoplasmic reticulum (Tada *et al.*, 1979; Movsesian *et al.*, 1984; Iwasa & Hosey, 1984; Kranias, 1985), in platelets PMA neither potentiates the phosphorylation of 22 kDa protein (Fig. 5) nor increases cyclic AMP even after thrombin treatment (Table 4). Consistent with this, adrenaline does not reverse the effect of PMA on the calcium transient after thrombin (Fig. 1). Furthermore, the platelet 22 kDa band is not similar to phospholamban, since it does not dissociate into 11 kDa monomer on boiling in SDS (results not shown), a characteristic test for phospholamban (Tada *et al.*, 1979; Movsesian *et al.*, 1984; Iwasa & Hosey, 1984; Kranias, 1985), and LePeuch *et al.* (1983) reported that anti-phospholamban antibody did not cross-react with platelet 22 kDa protein.

Thrombin also increases $^{45}Ca^{2+}$ uptake in saponin-treated platelets. In agreement with the effect of PMA on the calcium transient measured with fura-2 (Fig. 1), PMA added together with thrombin further increases calcium uptake into saponin-treated platelets. Since thrombin strongly stimulates protein kinase C (Nishizuka, 1986), this suggests that physiological activation of protein kinase C can also stimulate Ca^{2+} sequestration.

Connolly *et al.* (1986) reported that protein kinase C stimulated the phosphorylation and the activity of IP_3 phosphomonoesterase, and identified the enzyme as the 40–47 kDa protein. Molina y Vedia & Lapetina (1986) showed that phorbol ester enhanced the degradation of exogenous IP_3 in permeabilized platelets about 30%. Could our results be due to protein kinase C stimulation

of IP_3 degradation and the resultant decrease in IP_3-induced efflux? This seems unlikely because, firstly, we observed the same phenomenon with microsomal preparations in which the 47 kDa band fractionated almost entirely into the supernatant. Secondly, Brass *et al.* (1986) showed that guanosine 5′-[β-thio]diphosphate blocked the release of the small amount of IP_3 seen after saponin treatment of platelets, and we found that the analogue did not increase $^{45}Ca^{2+}$ uptake (results not shown). These observations support the conclusion that changes in IP_3 cannot account for all the effects of PMA in the saponin-treated system.

How, then, could PMA stimulate the sequestration of Ca^{2+}? We observe several phosphorylated bands after PMA treatment which may play a role similar to that of phospholamban in cardiac muscle. On the other hand, it is possible that the translocation of protein kinase C activity which we observe both in microsomes and in saponin-treated platelets could have a direct effect on sequestration, such as a change in the phospholipid environment (Uratsuji *et al.*, 1985). Another possibility is that a diffusible factor in proximity to the calcium-sequestering mechanism may be required. It will be important to determine whether translocated protein kinase C is associated with the intracellular membrane system. Since, when agonists bind to receptors on platelets, adenylate cyclase is inhibited (Katada *et al.*, 1985), regulation of calcium sequestration by this system probably does not play a role in returning calcium to baseline concentrations after agonist addition. On the other hand, protein kinase C is activated after the addition of agonists (Nishizuka, 1986). Our evidence suggests that in the platelet this may serve as a physiological signal for increased sequestration of internal Ca^{2+}. Finally, it appears that regulation of calcium sequestration in the platelet is not analogous to the regulation in heart sarcoplasmic reticulum.

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REFERENCES

Adunyah, S. E. & Dean, W. L. (1986) Cell Calcium **7**, 193–202

Authi, K. S., Evenden, B. J. & Crawford, N. (1986) Biochem. J. **233**, 707–718

Brass, L. F. & Joseph, S. K. (1985) J. Biol. Chem. **260**, 15172–15179

Brass, L. F., Laposata, M., Banga, H. S. & Rittenhouse, S. E. (1986) J. Biol. Chem. **261**, 16838–16847

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) J. Biol. Chem. **257**, 7847–7851

Chantler, P. D., Sellers, J. R. & Szent-Gyorgi, A. G. (1981) Biochemistry **20**, 210–216

Connolly, T. M., Lowing, W. J. & Majerus, P. W. (1986) Cell **46**, 951–958

Fox, J. E. B., Say, A. K. & Haslam, R. J. (1979) Biochem. J. **184**, 651–661

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450

Inagaki, M., Kawamoto, S. & Hidaka, H. (1984) J. Biol. Chem. **259**, 14321–14323

Iwasa, Y. & Hosey, M. M. (1984) J. Biol. Chem. **259**, 534–540

Käser-Glanzmann, R., Gerber, E. & Luscher, E. F. (1979) Biochim. Biophys. Acta **558**, 344–347

Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. & Jacobs, K. H. (1985) Eur. J. Biochem. **151**, 431–437

Kranias, E. G. (1985) Biochim. Biophys. Acta **844**, 193–199

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

LePeuch, C. J., LePeuch, D. A. M., Katz, S., Demaille, J. G., Hinckle, M. T., Enouf, R. B., Levy-Toledano, S. & Caen, J. (1983) Biochim. Biophys. Acta **731**, 456–464

Menashi, S., Authi, K. S., Carey, F. & Crawford, N. (1984) Biochem. J. **222**, 413–417

Molina y Vedia, L. M. & Lapetina, E. G. (1986) J. Biol. Chem. **261**, 10493–10495

Movsesian, M. A., Nishikawa, M. & Adelstein, R. S. (1984) J. Biol. Chem. **259**, 8029–8032

Naka, M., Nishikawa, M., Adelstein, R. S. & Hidaka, H. (1983) Nature (London) **306**, 490–492

Nishizuka, Y. (1986) Science **233**, 305–312

Pollock, W. K., Rink, T. J. & Irvine, R. F. (1986) Biochem. J. **235**, 869–877

Pollock, W. K., Sage, S. O. & Rink, T. J. (1987) FEBS Lett. **210**, 132–136

Rittenhouse, S. E. & Sasson, J. P. (1985) J. Biol. Chem. **260**, 8657–8660

Tada, M., Ohmori, F., Yamada, M. & Abe, H. (1979) J. Biol. Chem. **254**, 319–326

Tapley, P. M. & Murray, A. W. (1985) Eur. J. Biochem. **151**, 419–423

Uratsuji, Y., Nakanishi, H., Takeyama, Y., Kishimoto, A. & Nishizuka, Y. (1985) Biochem. Biophys. Res. Commun. **130**, 654–661

Watson, S. P. & Lapetina, E. G. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 2623–2626

Yoshida, K., Dubyak, G. & Nachmias, V. T. (1986) FEBS Lett. **203**, 273–278

Zavoico, G. B. & Feinstein, M. B. (1984) Biochem. Biophys. Res. Commun. **120**, 579–585

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