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Calcium transients in human platelets monitored by aequorin, fura-2 and quin-2: Effects of protein kinase C activation and inhibition

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Tumour-promoting phorbol esters and 1,2-dioctanoyl-sn-glycerol both induce calcium transients in platelets. However, these can only be detected in platelets loaded with aequorin, but not in those loaded with the fluorescent probes quin-2 and fura-2 presumably because of intracellular calcium buffering. Several effects induced by phorbol esters and diacylglycerols, including the rise in $(Ca^{2+})_i$, the stimulation of Na^+/H^+ transporter and the inhibition of the effects of thrombin alone on $(Ca^{2+})_i$ are potentently antagonised by staurosporine, a compound known to inhibit protein kinase C. Higher concentrations of staurosporine themselves inhibit the thrombin-induced calcium transient. Staurosporine inhibits the effects of phorbol esters and dioctanoyl glycerol with equal potency although the latter does not cause enzyme translocation of cytosolic protein kinase C to membranes. These results therefore suggest that some, if not all, the effects of protein kinase C activation can occur without translocation of the enzyme.

Hormone and drug inuced $(Ca^{2+})_i$ increase in various cells have been reported to be inhibited by prior addition of potent tumour-promoting phorbol esters presumably acting as stimulators of protein kinase C (PKC) (1-5). Phorbol esters alone have been reported to increase $(Ca^{2+})_i$, but this rise cannot be detected in cells loaded with of quin-2 (1,6) or the more recent

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Abbreviations: BCECF, 2', 7'-bis(carboxyethyl)-5,6-carboxyfluorescein; $(Ca^{2+})_i$, cytoplasmic free calcium concentration; DiC8, 1,2-dioctanoyl-sn-glycerol; EGTA, ethylene glycol bis (B-aminoethyl ether)-N,N,N',N'-tetraaceticacid; pH_i, cytoplasmic pH; PKC, Protein kinase C; TPA, 12-0-tetradecanoyl phorbol-13-acetate.

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fluorescent probe fura-2 (7). However a tumour promoter-mediated increase of the calcium signal can be monitored in aequorin loaded platelets (8). The primary event of PKC activation is thought to involve the translocation of PKC from the cytosol to the membrane (9,10). Recently we have shown that the inhibition of the agonist-mediated $(Ca^{2+})_i$ increase in a tumour cell line occurs at concentrations where the tumour promoters did not cause PKC translocation (1). Recently a potent and relatively specific inhibitor of PKC, staurosporine, has been described (11). We have therefore examined the effects of this compound on the $(Ca^{2+})_i$ increase induced by TPA and DiC8 in human platelets. We have further investigated the possibility that a putative inhibitor of PKC might restore agonist induced rises in $(Ca^{2+})_i$ in platelets treated with TPA and DiC8.

Materials and Methods

Human platelets were obtained from platelet rich plasma by centrifugation and loaded wit aequorin in EGTA containing solution using a modification of the method described by Vickers and Mustard (12). In brief, the procedures, which all were carried out at room temperature, involved resuspension of the platelets in 210 ul of a buffer containing 91 mm NaCl, 1.8 mM KCl, 8 mM $NaHCO_3$, 0.3 mM NaH_2PO_4 and 0.67 mg of glucose/ml (pH 7.35), to which 10 mM EGTA was added. To this platelet solution 5 uM ATP and 200 ug/ml aequorin was added after 10 min. After a further 5 min incubation, 2mM $MgCl_2$ and 42 ul of a buffer containing 2.74 M NaCl, 54 mM KCl, 238 mM $NaHCO_3$ and 8.4 mM NaH_2PO_4 were added to restore osmolarity. Following incubation of this solution for 15 min the platelets were immediately gelfiltered using a Sepharose CL-4B column which was preequilibrated with Hepes-Tyrode buffer (13). To the gelfiltered platelets 1 mM $CaCl_2$ was added and measurements performed after 15 min. $(Ca^{2+})_i$ was derived from luminescence measurements at 37°C as described by Johnson et al (13). Shortly before luminescence recordings were started, 3 mM EGTA and 400 ug/ml human fibrinogen were added to the platelet suspension. Luminescence and aggregation were measured simultaneously in a platelet-ionized calcium aggregometer (PICA, Coulter Inc.).

To compare different calcium-sensitive indicators, $(Ca^{2+})_i$ was also measured using quin-2 and fura-2. Platelets were incubated in platelet rich plasma at 37°C with 10 uM quin-2-acetoxymethylester for 10 minutes resulting in an intracellular quin-2 concentration of 0.8-1 mM and fluorescence measurements performed on a Perkin-Elmer 5LS spectrofluorimeter as described before (14). To incorporate fura-2, 1 uM fura-2-acetoxymethylester was added to platelets suspended in 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Hepes and 10 mM glucose pH 7.4 and incubated for 45 min (37°C). After a further wash to eliminate extracellular dye fluorescence was measured at 339 nm excitation and 500 nm emission at 37°C. The $(Ca^{2+})_i$ was calculated according to $(Ca^{2+})_i = Kd(F-Fmin)/(Fmax-F)$, where F represents the fluorescence in intact cells, Fmin the minimal fluorescnce after cell lysis in 10 mM EGTA, pH 8.5 and Fmax the maximal fluorescence upon lysing the cells in the presence of 1 mM $CaCl_2$. Cells were lysed with 50 uM digitonin. For calculations, 115 nM was used as dissociation constant, Kd, of quin-2 and 224 nM in the case of fura-2.

To investigate further effects of TPA and staurosporine pH_i was monitored with the use of the pH-sensitive fluorescent dye BCECF which was incorporated by incubation of $5x10^8$ platelets/ml in 10 mM Hepes 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM glucose (pH 7.15) with 15 uM

BCECF-acetoxymethylester vor 60 min at 37°C. After gelfiltration of the platelets on a Sepharose CL-2B column to eliminate extranous dye, fluorescence was recorded at 500 nm excitation and 530 nm emission at 30°C and pH_i was calibrated as described by Moolenaar et al (15).
For measurement of PKC activities platelets were prepared by centrifugation (14) and resuspended at a final concentration of $1\text{-}2\times10^9$ platelets/ml in a buffer containing 15 mM Tris-HCl (pH 7.4), 140 mM CaCl, 5.5 mM glucose and 0.2% bovine serum albumine. Following incubation with TPA or DiC8 for 10 min at 37°C, platelets were collected by centrifugation and washed once with ice cold suspension buffer, disrupted by ultrasound and subcellular fractions were prepared as described recently (1). PKC was determined as reported elsewhere in detail (16).

Staurosporine was kindly provided by CIBA-Geigy Ltd., Basel, Switzerland. Aequorin was purchased from Dr. J. Blinks, Mayo Clinics, Rochester, MA, USA. All other compounds were obtained commercially.

Results and Discussion

TPA as well as DiC8 cause a rapid rise in $(Ca^{2+})_i$ in aequorin-loaded platelets at low extracellular calcium concentration (fig.1b,f), as previously reported for TPA and other tumour promoters (8). No change in $(Ca^{2+})_i$ could be demonstrated in quin-2- or fura-2-loaded (data not shown) platelets. In platelets loaded with fura-2 as well as aequorin the TPA-induced $(Ca^{2+})_i$ rise was reduced (fig.1c). This effect was even more pronounced in quin-2-loaded platelets (data not shown). This difference between the two fluorescent dyes must be attributed to the higher calcium buffering capacity of the fluorescent probe quin-2. Pollock et al have also reported the abscence of a TPA-induced $(Ca^{2+})_i$ rise in fura-2-loaded platelets although in their study, performing the same incorporation procedure as in this, the

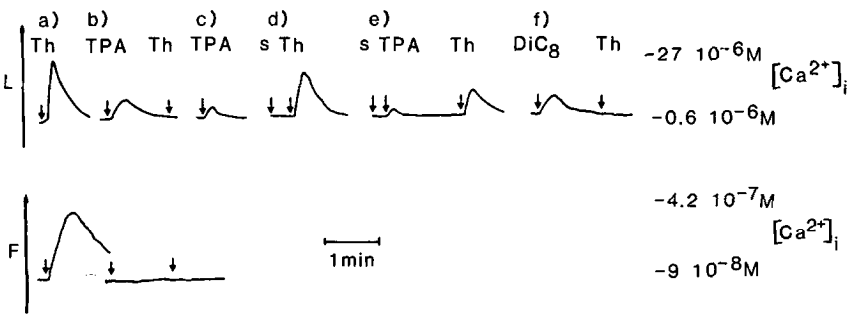


Figure 1. $(Ca^{2+})_i$ indicated by luminescence changes (L) to aequorin (upper trace) and fluorescence changes (F) to quin-2 (lower trace) in response to 0.3 U/ml thrombin (Th) and effects of 10^{-7} M TPA, 5×10^{-6} M DiC8 and 10^{-7} M staurosporine (s). Response to thrombin alone (a), to TPA with subsequent addition of thrombin (b), to TPA alone in fura-2-loaded platelets (c), to staurosporine with subsequent addition of thrombin (d) and to TPA and subsequent addition of thrombin following addition of staurosporine (e) and to DiC8 with subsequent addition of thrombin (f). Arrows indicate addition of agents.

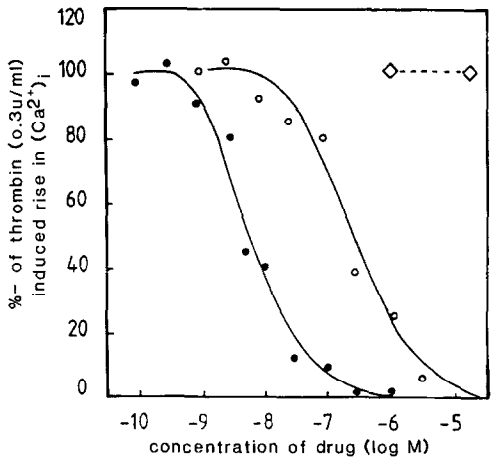


Figure 2. Concentration-response curve of TPA (closed circles) and DiC8 (open circles) on reduction of $(Ca^{2+})_i$ rise induced by 0.3 U/ml thrombin in aequorin-loaded platelets. 4α-phorbol 12,13-didecanoate (open squares) was ineffective at concentrations investigated. Measurements were performed after 2 min preincubation with TPA or DiC8.

concentration of intracellular fura-2 was as low as 30 uM (7). These results suggest that $(Ca^{2+})_i$ in an important cytoplasmic compartment can be monitored using aequorin; even low concentrations of currently used flurescent probes appear to buffer calcium transients in this compartment.

Preincubation of the platelets with both TPA and DiC8 markedly reduce the rise in $(Ca^{2+})_i$ induced by 0.3 U/ml thrombin in both aequorin- and fluorescent dye-loaded platelets (fig.1b,f) as well as $(Ca^{2+})_i$ response to ADP and adrenaline in the prescence of 1 mM extracellular calcium (data not shown). Although these agonists differ in the pathways by which they cause a rise in $(Ca^{2+})_i$, activation of PKC can prevent a $(Ca^{2+})_i$ response to all three agents. The inhibition of $(Ca^{2+})_i$ increase following 0.3 U/ml thrombin was concentration dependent with an IC_{50} of $7x10^{-9}$ M ($\pm$ 0.2;SD; n=4) for TPA, where DiC8 was less potent (IC_{50} $5x10^{-7}$ M; $\pm$ 0.3; n=3) (fig.2). While addition of TPA induces an increase in the activity of PKC in the membrane fraction and a simultaneous reduction in the cytosolic enzyme activity, implying a translocation of PKC from the cytosol to the plasma membrane, DiC8 (up to a concentration of 150 uM) causes no translocation (table 1). The data of this study support the hypothesis that PKC translocation is not essential for TPA-induced inhibition of responses to calcium-mobilising agonists, since the latter effect is observed at TPA concentrations too low to cause a significant translocation (1).

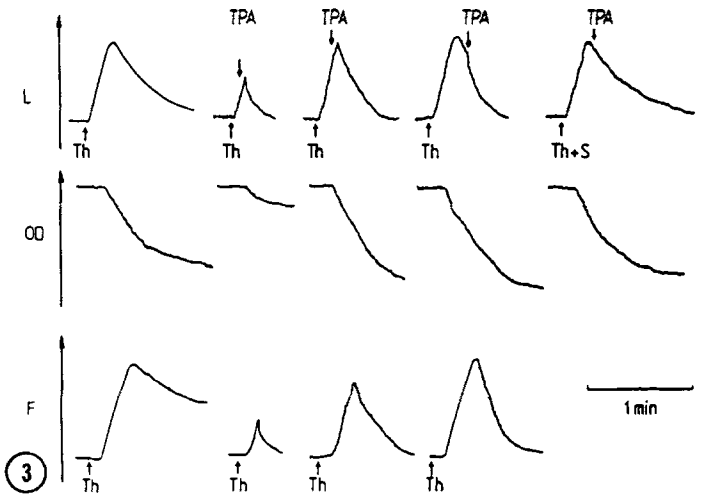
We have also examined the possibility that the effects of TPA could be reversed by PKC inhibitor staurosporine. While staurosporine alone had no effect on

Table 1. Distribution of protein kinase C in human platelets

Treatment	protein kinase C (units/mg)		protein kinase C (%-distribution)	
	cytosol	membranes	cytosol	membranes
Control	1000 ± 145	223 ± 85	81.8	18.2
TPA (1 uM)	180 ± 76	890 ± 202	16.8	83.2
DiC8 (150 uM)	914 ± 128	250 ± 133	78.5	21.5

aggregation and basal $(Ca^{2+})_i$ levels, addition of staurosporine inhibited the $(Ca^{2+})_i$ rise induced by TPA (fig.1d,e) with an IC_{50} of 2.7×10^{-8} M (± 0.1; n=5). Staurosporine also inhibited the response to DiC8 (3×10^{-7} M) with an IC_{50} which was not significantly different to TPA- (7×10^{-9} M) treated platelets. If staurosporine at concentrations greater than 10^{-8} M was added to platelets the rise in $(Ca^{2+})_i$ following the addition of 0.3 U/ml thrombin was reduced (fig. 1a,d) suggesting that mechanisms in addition to PKC inhibition may be operative. Such an additional mechanism would help to explain why in platelets treated with staurosporine and TPA (or DiC8) only part of the $(Ca^{2+})_i$ increase induced by thrombin in comparison to control platelets treated with thrombin alone can be restored (fig.1e).

When TPA was added within 10 seconds after the addition of 0.3 U/ml thrombin, the further thrombin-induced calcium rise in aequorin- and quin-2-loaded platelets was inhibited and aggregation reduced (fig.3). In contrast, if TPA was added at a later time the aggregation to thrombin was potentiated though the further $(Ca^{2+})_i$ rise in quin-2-loaded platelets was still inhibited. At all time points investigated, addition of TPA to thrombin-preactivated platelets increased the rate of the decay of the calcium signal. This effect can be antagonised by adding staurosporine before TPA supporting the hypothesis that this action of TPA is mediated by activation of PKC. These results also provide evidence that stimulation of PKC forms part of a control mechanism which depends upon a critical rise in $(Ca^{2+})_i$ (of about 300 nM as estimated in the quin-2-loaded platelets) and may protect against cellular calcium overload, possibly by stimulating calcium resequestration. This appears to be similar to the interaction observed between phorbol esters and platelet activating factor (17).



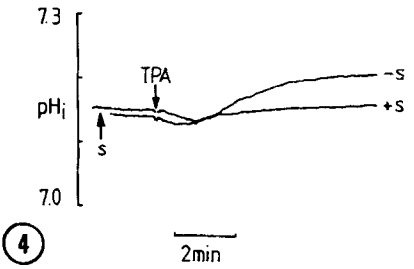


Figure 3. Effect of 10^{-7}M TPA on $(Ca^{2+})_i$ rise and fall following addition of 0.3 U/ml thrombin (Th). Same conditions as in figure 1. If $(Ca^{2+})_i$ rise exceeds a critical level, aggregation measured as changes in optical density (OD) is enhanced by synergistic action of thrombin and TPA. 10^{-8}M staurosporine (S) prevents this as well as the enhanced aggregation. Arrows indicate addition of agents.

Figure 4. Effect of 10^{-7}M TPA alone (-s) on pH_i in BCECF-loaded platelets. 10^{-7}M staurosporine (s) prevents alkalinization induced by TPA (+s). Arrows indicate addition of agents.

In numerous cells (eg 18), stimulation of PKC leads to an initial acidification which is followed by an alkalinization above physiological set points. While the initial acidification upon addition of TPA to BCECF-loaded platelets was minimal, alkalinization due to stimulation of Na^+/H^+ exchanger was more pronounced (fig.4). Addition of staurosporine prior to TPA inhibited this alkalinization further demonstrating that staurosporine is a potent inhibitor of PKC.

In conclusion, the results presented indicate that activation of PKC is accompanied by a rapid calcium transient which is inhibitory to further platelet activation by calcium mobilising agents and probably forms part of a mechanism protecting against cellular calcium overload. This rapid effect is followed by activation of the Na^+/H^+ exchanger. The effects induced by TPA and DiC8 are similar, suggesting that mechanisms additional to PKC translocation may be relevant, but can be counteracted by staurosporine. However, at least at higher concentrations, staurosporine elicits actions other than PKC inhibition; although a biphasic regulatory mechanism cannot be excluded comparable to that of the adenylate cyclase system (19).

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