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Protein kinase C stimulates dense tubular Ca^{2+} uptake in the intact human platelet by increasing the V_m of the Ca^{2+}-ATPase pump: stimulation by phorbol ester, inhibition by calphostin C

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The effects of protein kinase C (PKC) on Ca^{2+} transport were investigated in human intact platelets. The indicator quin2 was used to measure the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and to search for possible PKC effects on the Ca^{2+}-ATPase extrusion pump located in the plasma membrane. The Ca^{2+} indicator chlorotetracycline (CTC) was used to study PKC effects on the dense tubular Ca^{2+}-ATPase uptake pump. The activity of PKC was stimulated by phorbol 12-myristate 13-acetate (PMA) and was inhibited with calphostin C. Neither PKC activation nor inhibition had any effect on $[Ca^{2+}]_{cyt}$ or the Ca^{2+} extrusion pump. Substantial activation of the dense tubular pump was observed with PMA. In resting platelets bathed in 2 mM external Ca^{2+} giving $[Ca^{2+}]_{cyt}$ = 102–106 nM, activation of PKC by PMA (100 nM) increases the rate and extent of dense tubular Ca^{2+} uptake to 1.62 ± 0.35 and 1.25 ± 0.3 times control value (respectively). The V_m of the dense tubular pump was measured by using ionomycin to manipulate $[Ca^{2+}]_{cyt}$. It is shown that PMA increases the V_m by a factor of 1.7 ± 0.4 but has no effect on the K_m value (= 180 nM). An unexpected finding was that PKC activity supports a portion of the basal activity of the dense tubular Ca^{2+} pump in resting platelets. Preincubation with the inhibitor calphostin C (100 nM) decreases the rate and extent of dense tubular Ca^{2+} uptake in resting platelets by $38 \pm 5\%$ and $29 \pm 21\%$ (respectively). This is due to a $28 \pm 9\%$ decrease in the V_m of the dense tubular pump. This suggests that there is a low level of stimulation of dense tubular Ca^{2+} pump mediated by PKC in resting platelets.

Introduction

It is widely accepted that intracellular Ca^{2+} plays a key role in the activation of platelets. Protein kinase C (PKC) is an enzyme which plays a pivotal role in stimulus-response coupling in many cells [1]. Under physiological conditions, protein kinase C is stimulated by 1,2-diacylglycerol and by Ca^{2+} [2], although more recent evidence indicates that some subspecies of protein kinase C are not Ca^{2+}-dependent [3]. Protein kinase C can be stimulated by phorbol ester at basal cellular $[Ca^{2+}]_{cyt}$ level [4]. The influence of the protein kinase C system on Ca^{2+} transport has been an active area of interest. The present study describes the effects of PKC stimulation by phorbol 12-myristate 13-acetate (PMA) on the Ca^{2+} pumps and channels in the intact human platelet. This information will be applied to the more complicated problem of understanding PMA effects on agonist-generated Ca^{2+} transients.

Present evidence suggests that both increases in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and

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Abbreviations: cAMP, cyclic adenosine-3′,5′-monophosphate; cGMP, adenosine 3′,5′-cyclic monophosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PM, plasma membrane; ROC, receptor-activated channel; $[Ca^{2+}]_o$, the external Ca^{2+} concentration; $[Ca^{2+}]_{cyt}$, the free ('ionized') Ca^{2+} concentration in the cytoplasm; $[Ca^{2+}]_{dt}$, the free Ca^{2+} concentration in the dense tubular lumen; DT, dense tubules; quin2, 2-[[2[bis(carboxymethyl)amino]-5-methylphenoxy)methyl]-6-methoxy-8[bis(carboxymethyl)amino]quinoline; quin2/AM, tetraacetoxymethyl ester form of quin2; CTC, chlorotetracycline; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; A_{fast}, instantaneous CTC fluorescence change after Ca^{2+} addition; A_{slow}, time-resolved CTC fluorescence change after Ca^{2+} addition; R_{CTC}, 'CTC ratio' = A_{slow}/A_{fast}; V and V_m, the velocity and maximal velocity (respectively) of the Ca^{2+}-ATPase pump located in the dense tubular membrane; K_m, the $[Ca^{2+}]_{cyt}$ giving half-maximal rate of extrusion (V); $k_{leak,dt}$, rate constant for Ca^{2+} leakage from the dense tubular lumen; $k_{leak,PM}$ rate constant for Ca^{2+} leakage across the PM.

protein phosphorylation are necessary to achieve platelet activation. When human platelets are treated with PMA, a number of proteins are phosphorylated [5,6]. Among these are a 20 kDa protein identified as the myosin light chain [7,8] and a 47 kDa protein named pleckstrin [9,10]. The latter is considered a marker for protein kinase C activation [1,11]. Early studies reported evidence that treatment of platelets with PMA increases the sensitivity of platelet aggregation to Ca^{2+}. The observations were that PMA lowers the threshold agonist concentration for aggregation [12–14]. These same studies showed that the sensitization is correlated with an increase in agonist-stimulated secretion.

However, the various effects of PMA are seemingly contradictory. Other experimentation has shown that PMA can desensitize platelets to physiological activators. For example, Powling and Hardisty [14] showed that 1 nM PMA can completely inhibit aggregation induced by 10 nM platelet activating factor in the presence of 1 mM external Ca^{2+}. Several groups have reported that stimulation of PKC with PMA decreases the amplitude and shortens the duration of the transient increase in free cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) following stimulation with thrombin [15,16] and platelet activating factor [14,17]. Thus, PKC can also be considered to work against activation.

Studies in permeablized platelets and isolated dense tubules can be interpreted to suggest that the above-described suppression of Ca^{2+} transients may be attributable to increased dense tubular Ca^{2+} uptake. Yoshida and Nachmias [16] showed that PMA stimulates the rate of $^{45}Ca^{2+}$ uptake into the dense tubules of saponin-permeabilized platelets by a factor of 2–3. This finding was corroborated in a study using chlorotetracycline (CTC) to monitor dense tubular Ca^{2+} uptake in digitonin-permeabilized platelets [18]. Studies of $^{45}Ca^{2+}$ uptake by a microsomal fraction isolated from platelets showed that PMA increases the rate of Ca^{2+} uptake by a factor of 1.8 [19]. It was also shown that PMA and dilauroylglycerol stimulate Ca^{2+}-ATPase activity of internal plasma membrane [20].

The present study reports the effects of PKC stimulation and inhibition on the in situ behavior of the Ca^{2+}-ATPase pumps of the dense tubular system and plasma membrane. We manipulate the PKC of the intact platelet not only by stimulation but also inhibition. It has been recently reported that calphostin C, a newly isolated compound from *Cladosporium cladosporioides*, is a specific and potent inhibitor of PKC [21,22]. Calphostin C has several unique and useful features: It inhibits phorbol ester binding to PKC but does not compete with Ca^{2+} or phospholipid. It interacts with the regulatory domain of the enzyme [23]. It has been reported to be 1000-times more inhibitory to PKC than

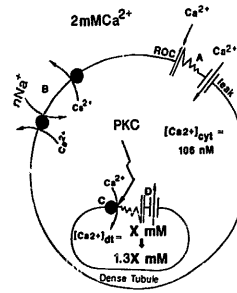

Fig. 1. Ca^{2+} handling systems of the platelet considered in the present study. Stimulatory effects of PKC on the tubular pump (denoted by zig-zag arrow) are shown in the present study. ROC denotes receptor-activated channel.

to other protein kinases such as cAMP-dependent kinase and tyrosine specific kinase [23]. Its inhibitory effect has been reported to be light dependent [24].

The present study is the fourth in a series describing the effects of second messengers on the Ca^{2+} handling in intact human platelets. These studies [25–27] are based on protocols using the cytoplasmic Ca^{2+} indicator quin2 [27] and the dense tubular Ca^{2+} indicator chlorotetracycline (CTC); Jy and Haynes [28,29] to study the plasma membrane Ca^{2+} extrusion pump and the dense tubular Ca^{2+} uptake pump, respectively. The Ca^{2+} handling systems in the human platelet are represented schematically in Fig. 1. Calcium homeostasis across the plasma membrane (PM) is maintained by the balance between passive leakage across the PM (Process A) and active extrusion by the PM Ca^{2+}-ATPase pump and the Na^+/Ca^{2+} exchanger (Process B). Similarly, dense tubular uptake depends on the balance between Ca^{2+}-ATPase-mediated uptake (Process C) and passive leakage across the dense tubular membrane [26,29].

We study these processes in the intact platelets by the use of fluorescent indicators for cytoplasmic and dense tubular Ca^{2+}. The kinetics of Ca^{2+} extrusion across the plasma membrane (PM) are studied in quin2-'overloaded' platelets by a protocol whereby the dye plays the dual role of $[Ca^{2+}]_{cyt}$ indicator and linear measure of the number of Ca^{2+} moved across the plasma membrane. First the cytoplasmically-trapped quin2 is saturated with Ca^{2+} by addition of 1 μM ionomycin to quin2 loaded platelets in the presence of 2 mM external Ca^{2+} ($[Ca^{2+}]_o$). Then further influx is halted by the addition of EGTA, and the kinetic characteristics of the active extrusion 'machinery' (Ca^{2+}-

ATPase pump and Na^+/Ca^{2+} exchanger; Process B, Fig. 1) are determined from the rate of Ca^{2+} removal vs. $[Ca^{2+}]_{cyt}$. Dense tubular Ca^{2+} uptake (Process C, Fig. 1) is studied using the fluorescent indicator chlorotetracycline (CTC) which monitors the free Ca^{2+} concentration in the dense tubules ($[Ca^{2+}]_{dt}$) [28,29]. The kinetics of the dense tubular uptake are analyzed in conjunction with $[Ca^{2+}]_{cyt}$ values which are obtained in parallel experiments with quin2. Ionomycin, together with programmed sequences of Ca^{2+} addition and removal, is used to manipulate the Ca^{2+} concentrations within cytoplasmic and dense tubular compartments.

Materials and Methods

Materials

Phorbol 12-myristate 13-acetate (PMA), EGTA, Hepes, quin2, glucose, chlorotetracycline (CTC) and forskolin were purchased from Sigma Chemical Co., St. Louis, MO. Ionomycin from Calbiochem, La Jolla, CA. Calphostin C from Kamiya Biomedical Co., Thousand Oaks, CA. The reagents used in the preparation of Tyrode's solution were supplied by Mallinkrodt Inc., Paris, KY.

Platelet isolation

Blood was drawn from normal donors into anticoagulant citrate dextrose. Washed platelets were prepared as described previously [29]. Cells were resuspended and experiments were performed in a nominally Ca^{2+}-free Tyrode's solution of the following composition: 138 mM NaCl/3 mM KCl/10 mM glucose/2 mM $NaHCO_3$/25 mM Hepes with the pH adjusted to 7.35. After isolation, platelets will lose Ca^{2+} as they approach a new steady state with this low $[Ca^{2+}]_o$. The cells are therefore referred to as being Ca^{2+}-depleted at the end of the isolation procedure. When EGTA was added to remove Ca^{2+}, an EGTA stock solution with pH preadjusted to 7.5–8.0 was used. This degree of alkalinity compensates the H^+ release resulting from Ca^{2+} binding to EGTA, so that the standard pH of 7.35 is maintained.

Fluorometry

All fluorescence measurements were made using a Perkin-Elmer fluorimeter (Model MPF-3L). The fluorescence instrumentation, the use of CTC and the description of its mechanism of response, the wavelengths and filters used, and precautions taken against possible artifacts for CTC and quin2 measurement have all been described [29]. CTC and quin2 excitation/emission wavelengths were 390/530 nm and 340/490 nm, respectively. A platelet concentration of $1.6 \cdot 10^7$ per ml was used for fluorometric experimentation. This was routinely measured turbidimetrically as an $OD_{600\,nm} = 0.20$ using a Beckman DB-G grating Spectrophotometer and occasionally verified with a Bright-line hemacytometer (American Optical). No dark or low-light experimentation was attempted with calphostin C.

Protocols measuring absolute rate of Ca^{2+} extrusion and rate of dense tubular Ca^{2+} uptake

Quin2 experimentation was carried out as described previously [25,30]. The quantitative measurement of the kinetics of Ca^{2+} extrusion was done by using quin2 overload/Ca^{2+} extrusion protocol [25,30]. In this experiment, platelets are incubated with 20 μM quin2/AM to obtain intracellular dye concentrations of about 3 mmol per liter cell volume, such that its Ca^{2+} buffer capacity is much larger than the Ca^{2+} buffer capacity intrinsic to the cytoplasm. A number of publications from this laboratory [18,28,31,32] have de-

TABLE I

Lack of effect of PMA on the kinetic parameters of the Ca^{2+} extrusion pump, the Na^+/Ca^{2+} exchanger the resting $[Ca^{2+}]_{cyt}$ and the rate constant for Ca^{2+} leakage ($k_{leak,PM}$) across the plasma membrane

Constant	Value		Unit
	control	PMA-treated	
V_m extrusion pump (PM) [a]	111 ±19	105 ±13	$\mu M\ min^{-1}$
	2.7± 0.5	2.5± 0.3	nmol $mg^{-1}\ min^{-1}$ [b]
K_m extrusion pump (PM) [a]	90	90	nM
Na^+/Ca^{2+} exchanger (PM) [c] (V_m/K_m ratio)	40 ±14	37 ±10	min^{-1}
	$(9.8 \pm 3.4) \cdot 10^{-4}$	$(9.1 \pm 2.5) \cdot 10^{-4}$	liter $mg^{-1}\ min^{-1}$ [b]
Resting $[Ca^{2+}]_{cyt}$ [d]	106 ±10	102 ±11	nM
$k_{leak,PM}$ $[Ca^{2+}]_o$ (PM) [c]	62 ± 9	58 ± 7	$\mu mol\ min^{-1}$

[a] Determined from experiments shown in Fig. 2 by analysis of eight experiments from different donors, Where indicated, PMA (100 nM) was preincubated with platelets for 15 min prior to the Ca^{2+} addition step.

[b] Rate expressed per mg membrane protein.

[c] The K_m of the Na^+/Ca^{2+} exchanger is sufficiently high that only the V_m/K_m ratio could be determined.

[d] The 'resting' $[Ca^{2+}]_{cyt}$ value was determined in the presence of 2 mM $[Ca^{2+}]_o$ as described in the text.

[e] The product of $k_{leak,PM}$, the rate constant for Ca^{2+} leakage across the PM, and when $[Ca^{2+}]_o = 2$ mM.

scribed the use of chlorotetracycline (CTC) as a measure of the free Ca^{2+} concentration in the dense tubular lumen ($[Ca^{2+}]_{dt}$). The measurement of the V_m and K_m of the dense tubular pump was carried out as described in a recent publication [26]. Since that protocol produced high $[Ca^{2+}]_{cyt}$ values which can enable mitochondrial Ca^{2+} uptake, 4 μM rotenone and 4 μg/ml oligomycin were added [26].

Statistics

All data were expressed as the mean ± S.D., except where noted. statistical analysis using Student's *t*-test were carried out with the aid of EPISTAT (copyright Tracy L. Gustafson). Best fit curves were produced using ASYSTANT (Macmillan Software Company).

Results

PMA has no effect on resting $[Ca^{2+}]_{cyt}$ or Ca^{2+} extrusion kinetics

The free concentration of cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) was measured under resting conditions in the presence and absence of 100 nM phorbol 12-myristate 13-acetate (PMA). As described previously [29,30], resting values of $[Ca^{2+}]_{cyt}$ are determined by the balance between Ca^{2+} influx via the passive leak (Process A, Fig. 1) and active Ca^{2+} extrusion across the PM (Process B, Fig. 1). In these particular experiments platelets were loaded at an 'indicator' level of quin2: The platelets were preincubated with 5 μM quin2/AM to achieve cytoplasmic quin2 concentrations of 0.82 ± 0.23 mM. A platelet suspension was preincubated for 15 min with and without 100 nM PMA, then 2 mM Ca^{2+} was added and $[Ca^{2+}]_{cyt}$ was measured after an additional 15 min. Six paired experiments gave resting $[Ca^{2+}]_{cyt}$ values of 106 ± 10 (S.D) nM for control and 102 ± 11 nM for PMA treatment. This result is entered in Table I.

The lack of a PMA effect on $[Ca^{2+}]_{cyt}$ suggests that neither the extrusion process nor the passive influx is altered by PKC stimulation. This was directly tested, by studying active Ca^{2+} extrusion into a Ca^{2+}-free medium with quin2-overloaded platelets according to a protocol described previously [30]. Fig. 2 presents a typical experiment comparing the progress curves for Ca^{2+} extrusion from PMA-treated and control platelets. The experiment gives no indication of any effect of PMA treatment on the extrusion process. The kinetic constants of the extrusion systems were as described previously [30]. Table I presents the results of analysis of 8 repetitions of this experiment. The table shows that PMA is without effect on the V_m or K_m of the plasma membrane (PM) Ca^{2+} extrusion pump and the has no effect on the V_m/K_m ratio of Na^+/Ca^{2+} exchanger. The rate constant for passive Ca^{2+} leak across the plasma membrane ($k_{leak,PM}$) can be determined from

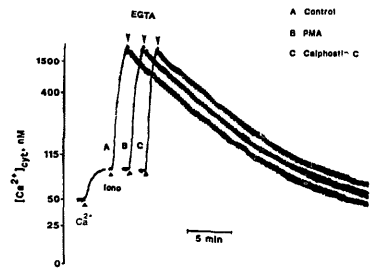

Fig. 2. Lack of effect of 100 nM PMA or 100 nM calphostin C on the kinetics of Ca^{2+} extrusion. The experimentation was carried out according a protocol described previously [30]. Quin2-overloaded platelets (entrapping approx. 3.6 mmol quin2/liter cell volume) were incubated with 2 mM external Ca^{2+} for 15 min. Where indicated 1 μM ionomycin was added causing rapid Ca^{2+} influx. At the point where the cytoplasmic quin2 was approx. 98% saturated, external Ca^{2+} was removed by EGTA complexation. The decrease in fluorescence is proportional to the number of Ca^{2+} removed from the cytoplasm by the pump and exchanger. Calculated $[Ca^{2+}]_{cyt}$ values are given by the non-linear scale on the left. Control experiments (not shown) were carried out with CTC to confirm that the dense tubules did not make a significant contribution to Ca^{2+} removal under these conditions (Fig. 3 of Ref. 25). In the PMA- and calphostin C-treated cases, these agents were added 15 and 20 min before the Ca^{2+} addition, respectively.

the kinetic constants of the extrusion system and the steady-state value of $[Ca^{2+}]_{cyt}$ [25]. Since none of the above values changes with PMA treatment, $k_{leak,PM}$ is also unchanged.

PMA increases the rate of uptake and resting level of dense tubular Ca^{2+} concentration

Our previous studies [26,28,29,33] have shown that the chlorotetracycline (CTC) fluorescence signal is a linear measure of the free Ca^{2+} concentration in the dense tubular lumen ($[Ca^{2+}]_{dt}$). Fig. 3 is a typical CTC experiment showing that PMA increases the rate and extent of dense tubular Ca^{2+} uptake in resting platelets. Fig. 3 shows that PMA increases both the initial rate and maximal extent of dense tubular Ca^{2+} uptake in resting platelets bathed in 2 mM external Ca^{2+}. In the presented experiment, 100 nM PMA was preincubated with the platelets 15 min before the indicated addition of external Ca^{2+}. The data of Table II show that this represents a maximally effective concentration and preincubation time.

Eight repetitions of the experiment of Fig. 3 show that PMA enhances the initial rate and maximal extent of dense tubular Ca^{2+} uptake in the 'resting' platelet by a factors of 1.62 ± 0.35 and 1.25 ± 0.30, respectively. These data are presented in Table III.

TABLE II

Preincubation time and concentration dependence of the PMA-induced stimulation of the rate and maximal extent of dense tubular Ca^{2+} uptake

The experiment of Fig. 2 was performed at variable PMA concentration and preincubation time (minutes before Ca^{2+} addition). Where indicated, 500 nM ionomycin was added simultaneously with Ca^{2+}. All of the presented ($n = 6$–8) data are expressed in terms of 'CTC units' whereby the observed rate and maximal extent are divided by A_{fast}, the amplitude of the instantaneous phase. The latter is a constant which reflects only the area of plasma membrane outer leaflet exposed to the external medium [26,28,29,33].

[PMA] (nM)	Preincubation time (min)	Control		Ionomycin-treated	
		initial rate (units/min)	max. extent (R_{CTC}) (units)	initial rate (units/min)	max. extent (R_{CTC}) (units)
0	–	0.12 ± 0.02	0.20 ± 0.03	0.85 ± 0.20	0.80 ± 0.09
25	15	0.15 ± 0.05 [a]	0.22 ± 0.04 [a]	1.16 ± 0.25 [a]	0.84 ± 0.05 [a]
100	1	0.17 ± 0.03 [a]	0.21 ± 0.06	1.10 ± 0.18 [a]	0.88 ± 0.05 [a]
100	15	0.20 ± 0.05 [a]	0.25 ± 0.08 [a]	1.40 ± 0.18 [a]	1.04 ± 0.12 [a]
100	45	0.20 ± 0.04 [a]	0.25 ± 0.06 [a]	1.40 ± 0.22 [a]	1.05 ± 0.07 [a]
500	15	0.20 ± 0.05 [a]	0.25 ± 0.07 [a]	1.35 ± 0.20 [a]	1.04 ± 0.06 [a]

[a] Significantly greater than control ($P < 0.05$).

PMA increases the V_m of dense tubular Ca^{2+} pump

As developed previously [26], measured levels of dense tubular Ca^{2+} uptake depend on the balance between active uptake and passive leakage as described in the following equation:

$$V = V_{m,dt}\frac{[Ca^{2+}]_{cyt}^{1.4}}{\left(K_m^{1.4} + [Ca^{2+}]_{cyt}^{1.4}\right)} - k_{leak,dt}[Ca^{2+}]_{dt} \qquad (1)$$

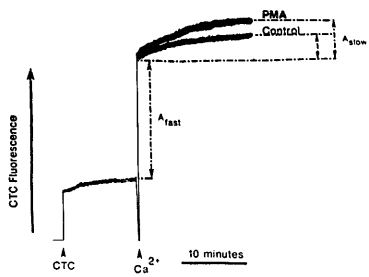

Fig. 3. Effect of PMA (100 nM) on the rate of dense tubular Ca^{2+} uptake in intact platelets monitored with CTC fluorescence. The standard protocol of Jy and Haynes [28,29] was used. Washed Ca^{2+}-depleted platelets ($1.6 \cdot 10^7$/ml) were introduced into the cuvette and 10 μM CTC was added together with 4 μM rotenone and 4 μg/ml oligomycin. Where indicated, PMA was also added at this time. Ca^{2+} (2 mM) was added where indicated. Active uptake is measured by the time resolved increase in CTC fluoresence after registration of the instantaneous increase (amplitude is denoted A_{fast}) after Ca^{2+} addition. The amplitude of dense tubular uptake is denoted A_{slow}. Quantitation of $[Ca^{2+}]_{dt}$ is by means of the 'CTC ratio' (R_{CTC}) which is calculated as A_{slow}/A_{fast} as illustrated in the figure.

where $V_{m,dt}$ is the maximal velocity of the dense tubular pumps at saturating $[Ca^{2+}]_{cyt}$, where K_m is the $[Ca^{2+}]_{cyt}$ for half-maximal velocity, where the exponent 1.4 is the Hill coefficient for the process and where $k_{leak,dt}$ is the rate constant for passive Ca^{2+} leakage of Ca^{2+} across the dense tubular membrane [26]. It follows that PMA effect of Fig. 3 and Table III could be attributed to either an increased V_m, a decreased K_m, of dense tubular Ca^{2+} pump or decreased k_{leak} of dense tubules. We have shown [26] that $V_{m,dt}$ can be measured by an 'ionomycin challenge' protocol, in which the experiment of Fig. 3 is repeated with ionomycin at concentrations chosen to raise $[Ca^{2+}]_{cyt}$ to saturating levels without seriously short-circuiting the dense tubular pump.

TABLE III

Stimulation and inhibition of rate and extent of dense tubular Ca^{2+} uptake by PMA and calphostin C

PKC was stimulated in Ca^{2+}-depleted platelets by 15 min preincubation in the cuvette with 100 nM PMA and inhibited by 20 min preincubation with 100 nM calphostin C. CTC (10 μM), 4 μM rotenone and 4 μg/ml oligomycin were also present during the preincubation. Then 2 mM Ca^{2+} was added to initiate dense tubular uptake which was monitored by CTC fluorescence. Where indicated, 500 nM ionomycin was added simultaneously. The initial rate ($V_{initial}$) and CTC ratios (R_{CTC}) were determined. The table presents the ratio of these quantities for the treated vs. control cases. The presented values are means for 6–8 paired experiments ± S.D.

[Iono] (nM)	PMA-treated		Calphostin C-treated	
	V_{PMA}/V_{cont}	$R_{CTC,PMA}/R_{CTC,cont}$	V_{PMA}/V_{cont}	$R_{CTC,PMA}/R_{CTC,cont}$
0	1.62 ± 0.35	1.25 ± 0.3	0.62 ± 0.05	0.71 ± 0.21
500	1.70 ± 0.40 [a]	1.35 ± 0.22	0.72 ± 0.09 [b]	0.85 ± 0.15

[a] Identical to ratio of $V_{m,PMA}/V_{m,cont}$.

[b] Identical to ratio of $V_{m,calphostinC}/V_{m,cont}$.

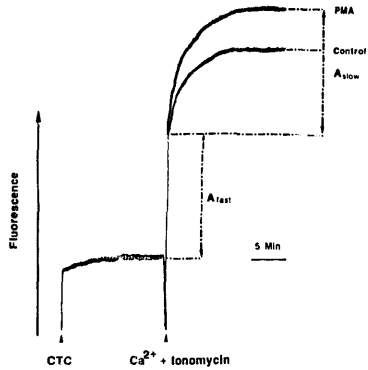

Fig. 4. Effect of PMA on Ca^{2+} uptake at high $[Ca^{2+}]_{cyt}$ values elicited by 500 nM ionomycin. The experiment is identical to that of Fig. 3 except that ionomycin was added simultaneously with the Ca^{2+}.

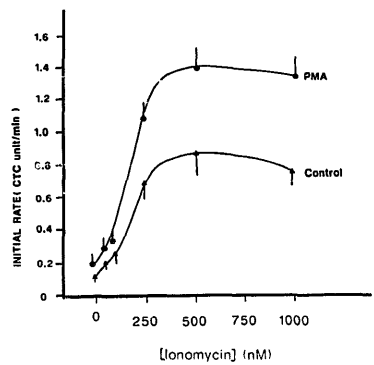

Fig. 5. Effect of ionomycin on initial rates of dense tubular Ca^{2+} uptake measured by CTC fluorescence in the presence and absence of PMA. The experiment was identical to that of Fig. 3 except that the ionomycin concentration was varied. The initial rate, determined over the first 10 s, is expressed as $V = (dFl/dt)/A_{fast}$. The data are the average (±S.D.) of eight paired experiments. The ascending portion of the curve reflects the ionomycin-induced increase in $[Ca^{2+}]_{cyt}$, the small decline phase observed between 500 nM and 1000 nM ionomycin reflects some short-circuit of dense tubular uptake by the ionophore.

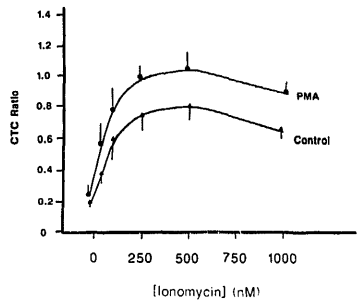

Fig. 6. Effect of ionomycin on maximal extent of dense tubular Ca^{2+} uptake measured by CTC fluorescence in the presence and absence of PMA. The 'CTC ratio' is the ratio of amplitudes of the slow phase to the instantaneous phase (A_{fast}/A_{slow}) following Ca^{2+} addition to platelets. It is proportional to $[Ca^{2+}]_{dt}$. The data are from the same experiments as in Fig. 5.

Fig. 4 shows the PMA effect on the initial rate and maximal extent of dense tubular Ca^{2+} uptake at 500 nM ionomycin, a concentration which had previously been shown to be optimal for this purpose [26]. Fig. 5 confirms that 500 nM ionomycin has produced sufficient elevation of $[Ca^{2+}]_{cyt}$ to allow the V_m of the pump to be expressed. This is also reflected in Fig. 6 which shows the maximal extent of uptake in the same experiments. The above data show that PMA increases the V_m of the dense tubular pump by a factor of 1.70 ± 0.40. These data and the aforementioned data are presented in Table III.

PMA does not affect the K_m of the dense tubular pump

The effect of PMA on the K_m of dense tubular pump can be demonstrated through application of Eqn. 1, together with the knowledge of V_m, the initial rate and $[Ca^{2+}]_{cyt}$ at the instant that 2 mM Ca^{2+} is added [26]. In Ca^{2+}-depleted platelets $[Ca^{2+}]_{cyt} \approx 50$ nM and the resting $[Ca^{2+}]_{dt}$ is very low. Thus, the second term of Eqn. 1 does not contribute to the kinetics of dense tubular Ca^{2+} uptake in the initial phase. For the dense tubular pump operating unopposed by leakage we have derived [26] from Eqn. 1 the following equation,

$$K_m = [Ca^{2+}]_{cyt}\left(\frac{1-X}{X}\right)^{1/1.4} \quad (2)$$

where X is defined as the degree of saturation of the pump with cytoplasmic Ca^{2+} ($X = V/V_m$) The V and V_m values are taken from Fig. 5 for 0 nM and 500 nM ionomycin, respectively. Using this and the above-men-

TABLE IV

Lack of effect of PMA on kinetics of the dense tubular Ca^{2+} pump and Ca^{2+} leak across the dense tubular membrane

$[Ca^{2+}]_{cyt}$ values are for the 'resting' state in the presence of 2 mM Ca^{2+} [25]. The V_m values (CTC units/min) are calculated from the initial rate at [Iono] = 0.5 μM as described in the text. The K_m values are calculated as described in the text. $[Ca^{2+}]_{dt}$ is the measured R_{CTC} value (Fig. 6). X is the degree of saturation of the pump in steady state, calculated from Eqn. 3 of Ref. 26, using steady state $[Ca^{2+}]_{cyt}$ and K_m value. $R_{CTC,max}$ (CTC units) is the experimental value for 0.5 μM ionomycin or the theoretical value (R_{CTC}/X) for no ionomycin. k_{leak+1}/V_m stands for $(k_{leak} + k_{iono}[Iono])/V_m$ (min^{-1}) which is identical to X/R_{CTC} (Eqn. 6 of Ref. 26). k_{leak+1}/V_m (CTC units^{-1}) was calculated from k_{leak}/V_m using $k_{iono} = 0.34\ k_{leak}$ (μM^{-1}) [26]. Finally, k_{leak} (min^{-1}) was calculated from the preceding column using the appropriate V_m value.

State	[Iono] (μM)	$[Ca^{2+}]_{cyt}$ (nM)	K_m (nM)	V_m (CTC units)	$[Ca^{2+}]_{dt}$	X (CTC units)	$R_{CTC,max}$	$\frac{k_{leak+1}}{V_m}$	$\frac{k_{leak}}{V_m}$	k_{leak} (min^{-1})
Control	0	106	180	0.85	0.20	0.30	0.67	–	1.27	1.10
Control	0.5	≥ 1000	180	0.85	0.80	1.00	0.80	1.25	1.10	0.94
PMA	0	102	182	1.4	0.25	0.3	0.83	–	1.03	1.40
PMA	0.5	≥ 1000	182	1.4	1.08	1.00	1.08	0.93	0.79	1.11

tioned $[Ca^{2+}]_{cyt}$ value (50 nM), we obtain the following K_m, values: 180 nM, control; 182 nM, PMA-treated. Thus, PMA is without influence on the K_m of the dense tubular Ca^{2+} pump. We note that the derived control value is in excellent agreement with the K_m value obtained in digitonin-permeabilized platelets [29]. The K_m values are entered in Table IV.

Lack of effect of PMA on the k_{leak} of dense tubules

We have previously shown that the values of $k_{leak,dt}$ can be calculated from the values of $[Ca^{2+}]_{dt}$ in steady-state at rest [26]. The calculation is analogous to the determination of $k_{leak,PM}$ presented in an earlier section. Table IV shows that PMA has no significant effect on $k_{leak,dt}$.

PMA and cAMP have no additive effect on the V_m of dense tubular Ca^{2+} uptake

Like PMA, cAMP has been shown to increase the V_m but have no effect on K_m of dense tubular Ca^{2+} uptake [26]. It is thus of interest to determine whether cAMP could produce any additional effect in the presence of a maximally-effective PMA concentration. The experimentation presented in Table V shows that this is not the case.

TABLE V

Lack of additional effect of forskolin after PMA activation

Ionomycin challenge experiments measuring dense tubular Ca^{2+} uptake were carried out as in Fig. 4 except that PMA (100 nM) was preincubated with platelets with or without forskolin (10 μM) for 15 min prior to addition of ionomycin and Ca^{2+}.

Agent	Initial rate (units/min)	Max. extent (units)
Control	0.82 ± 0.18	0.80 ± 0.09
PMA	1.38 ± 0.12 [a]	1.04 ± 0.12 [a]
Forskolin	1.36 ± 0.14 [a]	1.04 ± 0.10 [a]
PMA + forskolin	1.37 ± 0.21 [b]	1.04 ± 0.14 [b]

[a] Significantly different from control ($P < 0.05$).

[b] Not significantly different from PMA ($P > 0.1$).

Effect of calphostin C on the Ca^{2+} transport processes

To further study the effects of PKC on the Ca^{2+} transport, we made use of the newly isolated, highly specific PKC inhibitor, calphostin C. The inhibitor has no effect on the resting value of $[Ca^{2+}]_{cyt}$ in the presence of 2 mM external Ca^{2+}. The results from six paired experiments with 100 nM calphostin C preincubation gave an average $[Ca^{2+}]_{cyt}$ value of 102 ± 8 nM vs. 100 ± 10 for control ($P > 0.1$). These values are entered in Table VI. The lack of effect on resting $[Ca^{2+}]_{cyt}$ is not unexpected, since PMA was also un-

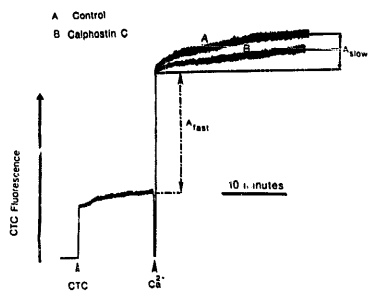

Fig. 7. Effect of calphostin C on the rate and extent of dense tubular Ca^{2+} uptake in resting platelets. The experiment was carried out as in Fig. 3 except that the platelets were preincubated with 100 nM calphostin C for 20 min before the Ca^{2+} addition. The inhibitor did not change the Ca^{2+} sensitivity of CTC as demonstrated by the lack of effect on the amplitude of the fast phase of CTC fluorescence in resting platelets. Also, the experiment was repeated with 500 nM calphostin C (not shown) and progress curves were identical to those observed for 100 nM calphostin C.

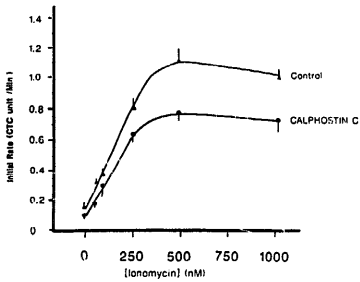

Fig. 8. Effect of ionomycin on initial rate of dense tubular Ca^{2+} uptake measured by CTC fluorescence in the presence and absence of calphostin C. The standard protocol was used as shown in Fig. 3. Platelets were preincubated with 100 nM calphostin C for 20 min.

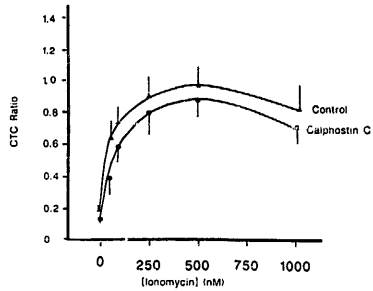

Fig. 9. Effect of ionomycin on maximal extent of dense tubular Ca^{2+} uptake measured by CTC fluorescence in the presence and absence of calphostin C. The standard protocol was used as shown in Fig. 3. Platelets were preincubated with 100 nM calphostin C for 20 min.

to change this quantity. Calphostin C also had no effect on the Ca^{2+} extrusion pump. This is shown in Fig. 2 and is tabulated in Table VI. The progress curves for extrusion in the control, calphostin C and PMA-treated cases are superimposable.

Calphostin C did have a substantial effect on dense tubular Ca^{2+} uptake. Fig. 7 is a typical experiment showing that 100 nM calphostin C decreases both the initial rate and extent of dense tubular Ca^{2+} uptake in resting platelets. The experiment was repeated six times and the data are entered in the right-hand portion of Table III. The data indicate that calphostin C inhibits the rate of Ca^{2+} uptake in resting platelets by 38% and inhibits the extent by about 30%. We repeated the ionomycin challenge protocol described for PMA in Figs. 5 and 6 to determine whether the calphostin C effect was on the V_m or K_m. Fig. 8 shows that a maximally-effective concentration of calphostin C decreases the V_m of the dense tubular pump by 30%. Fig. 9 shows that calphostin C has a similar effect on the maximal extent of Ca^{2+} uptake (15% decrease). We calculated the K_m values for the data at zero ionomycin by the method described in the previous section. Calphostin C was without effect on the K_m (203 ± 12 nM, $n = 5$ vs. 184 ± 36 nM, control, $n = 7$, $P > 0.1$). The results are entered in Table VI.

Discussion

The principal results of this study are summarized in Table VI. Protein kinase C stimulation by PMA increases the rate and extent of Ca^{2+} uptake by the dense tubular pump but does not affect the Ca^{2+} transport mechanisms in the plasma membrane. Combining this with our previous findings shows that each of the three protein kinases has a different pattern of effects on the PM vs. dense tubular pump: Protein kinase A stimulates both pumps [25,26], protein kinase and stimulates the PM pump only [27] and protein kinase C stimulates the dense tubular pump only. It is interesting to note that the maximally PKC-stimulated dense tubular pump can not be further stimulated by protein kinase A (forskolin, present study). An unex-

TABLE VI

Summary of kinetic constants of the Ca^{2+} pumps in three states of PKC activation

The presented values are means for 6–8 paired experiments ± S.D. or estimate of uncertainty (for $k_{leak,dt}$).

State	Plasma membrane				Dense tubular membrane			
	V_m (μM-min^{-1})	K_m (nM)	k_{leak} (min^{-1})	$[Ca^{2+}]_{cyt}$ (nM)	V_m (CTC units)	K_m (nM)	k_{leak} (min^{-1})	$[Ca^{2+}]_{dt}$ (CTC units)
Inhibited	111 ± 19	90 ± 10	31 ± 4	102 ± 8	078 ± 0.07 [a]	203 ± 12	1.2 ± 0.11	0.15 ± 0.01 [a]
Basal	111 ± 15	90 ± 10	31 ± 4	103 ± 10	0.95 ± 0.10	182 ± 22	1.2 ± 0.11	0.20 ± 0.02
Stimulated	105 ± 13	90 ± 9	29 ± 3	102 ± 8	1.40 ± 0.18 [a]	182 ± 8	1.4 ± 0.12	0.25 ± 0.08 [a]

[a] Significantly different from basal (control) ($P < 0.01$).

pected observation of the present study is that PKC is responsible for a portion of the dense tubular Ca^{2+} uptake in the reating state of *untreated* platelets. This observation was made using the new specific PKC inhibitor calphostin C.

Mechanism of PMA stimulation of the dense tubular pump

Our findings show that PMA increases the rate and extent of dense tubular Ca^{2+} uptake in the intact platelet. This is in agreement with previous finding of PMA stimulation of Ca^{2+}-ATPase [20] and Ca^{2+} uptake in platelet microsomal fractions [19] and with observation with detergent-permeabilized platelets [16,18]. The present study shows that the stimulation is expressed as a V_m effect. The K_m is not altered. The effect of PMA on dense tubular Ca^{2+} pump does not seem to be mediated by increasing the cAMP level because PMA has been reported to have no effect on the latter [19].

It is of interest to consider the stimulation of the dense tubular pump in relation to what is known about regulatory phosphorylation of platelet proteins induced by PKC. In human platelets it has been known that cAMP-dependent phosphorylation of a 22 kDa protein stimulates Ca^{2+} uptake of dense tubules [34,35]. This protein has physicochemical similarity to phospholamban [36–38], a 22 kDa protein which regulates the activity of the Ca^{2+}-ATPase pump of the cardiac muscle sarcoplasmic reticulum. Phospholamban is phosphorylated by a cAMP-dependent protein kinase [37,38], by calmodulin [39], and by PKC [40]. All three agents are capable of increasing the rate of the cardiac SR Ca^{2+} pump in a phospholamban-mediated process [40].

Yoshida and Nachmias [16,41,42] have addressed the question of whether phosphorylation of the 22 kDa protein is the mechanism by which PMA stimulates dense tubular Ca^{2+} uptake and have concluded that it is not. Their reasoning was based on the finding that 20 nM PMA produced less phosphorylation of a 23 kDa protein than of a 27 kDa protein. However, the gel patterns of ^{32}P-labelled proteins did show the presence of a 23 kDa label led protein in the unstimulated state [16,41]. Furthermore, the presented gels did (in our judgement) show some increase in phosphorylation of a 23 kDa protein with treatment of intact platelets with 20 nM PMA. PMA-stimulated phosphorylation of proteins in the 20–22 kDa range has also been reported by others [6]. In light of our finding of PKC-mediated stimulation of the dense tubular Ca^{2+} uptake in the *basal* state, and considering our observation that PKA and PKC stimulation of uptake are not additive, we believe that this question is worthy of further consideration.

The phosphorylation/non-phosphorylation pattern fits well with our finding that PKC does not stimulate the rate of the Ca^{2+} extrusion pump located in the PM. By analogy to the cardiac sarcolemmal pump, the expected molecular weight of this protein would be 130 kDa [43]. As we have previously noted, a 130 kDa protein in platelets is phosphorylated by both cAMP and cGMP [44], agents which stimulate the Ca^{2+} extrusion pump [25,27]. Published gel patterns of PMA-stimulated phosphorylation do not show labelling of a protein in this range [6].

Relationship between PKC-dependent stimulation of the dense tubular Ca^{2+} pump and pro- and anti-activation effects of PMA

A detailed consideration of PMA effects on platelet Ca^{2+} handling is important since PKC is stimulated by diacylglyceride during the course of platelet activation. As described in the Introduction, both pro-activation and anti-activation effects of PMA have been reported.

PMA-stimulated protein phosphorylation is important to the pro-activation effects of this agent. As presented by others, protein phosphorylation is a sufficient explanation for the pro-activation effects [11-14,47] at low agonist concentrations or low $[Ca^{2+}]_{cyt}$. Important protein substrates are the 20 kDa myosin light chain [7,8] and 47 kDa pleckstrin [9], which have been linked to the shape change and serotonin release reactions [5,45,46].

The present findings are in agreement with the anti-activation effects of PMA. Although PMA increases the resting level of Ca^{2+} in the dense tubules (present study), it also inhibits IP_3 formation [48,49] and stimulates the degradation of IP_3 [50]. Apparently the effect on IP_3 outweighs the effect on $[Ca^{2+}]_{dt}$. A decrease in the amplitude of the IP_3 signal would be sufficient to explain the finding that PMA decreases the amplitude of the Ca^{2+} transients evoked by thrombin, ADP and PAF [14,15]. Other workers have reported that PMA increases the rate at which the calcium signal returns to the baseline [14,15,17]. Our finding of increased rates and extents of dense tubular Ca^{2+} uptake is a sufficient explanation for this. Our study offers no support for suggestions that extrusion across the PM membrane is stimulated by PMA *. It would be of interest to determine whether diacylglycerol released during activation affects the time course of the Ca^{2+} transient.

* Pollock et al. [52] showed that PMA increases the rate of fluorescence decrease after adding fura-2 loaded platelets to an EGTA-containing solution ($[Ca^{2+}]_o < 10^{-8}$ M) and then adding ionomycin. However, since the ionomycin concentration was not varied, it is not clear that dense tubular uptake was completely short-circuited under their conditions.

It is possible that the PKC effect on dense tubular Ca^{2+} levels serves as a sort of 'integrator' of previous stimulation by agonists. Our finding of partial inhibition of dense tubular uptake by calphostin C suggests that basal PKC activity partially supports the dense tubular Ca^{2+}-ATPase pump, and further supports resting (and releasable) $[Ca^{2+}]_{dt}$. Relevant to this are published gel patterns showing some phosphorylation of protein in the 20–23 kDa range in the basal state of resting platelets [16,41]. Phosphorylation and 'activation' in the basal state may prove to be a result of basal activity of PKC or of basal activity of the phospholipase C which stimulates it. This is interesting in light of the finding that the platelet contains 3 or 4 isoforms of PKC and that binding to membrane increases with activation [51]. A better understanding of these relationships must await a systematic study of calphostin C effects on protein phosphorylation in the human platelets.

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