Rapid Publication

Phorbol Myristate Acetate Stimulates ATP-dependent Calcium Transport by the Plasma Membrane of Neutrophils

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Abstract. We studied the effect of phorbol myristate acetate (PMA) on the plasma membrane ATP-dependent calcium pump in neutrophils. Plasma membrane-enriched fractions ("podosomes") from PMA-stimulated guinea pig neutrophils exhibited a twofold stimulation of ATP-dependent calcium transport when compared with control podosomes. The stimulatory effect was rapid (beginning less than 2 min after exposure to PMA) and reached maximal values within 5 min. PMA increased the maximum velocity but not the affinity of the calcium pump for Ca^{++}.

Pump activation was not preceded by a rise in cytosolic free calcium concentration $[Ca^{++}]_i$, as assessed by the intracellularly trapped fluorescent calcium indicator Quin 2, but instead slightly lowered $[Ca^{++}]_i$ and prevented the rise in $[Ca^{++}]_i$ normally induced by the chemotactic peptide formyl-methionyl-leucyl-phenylalanine.

These results suggest that the calcium pump in the plasma membrane of neutrophils may be stimulated by calcium-independent pathways, and that this activation could be one of the earliest events mediating some of the effects of phorbol esters.

Introduction

Phorbol esters are potent tumor promoters (1), inducing the expression of transformation-like properties in a variety of undifferentiated cell lines in vitro (2, 3). In addition, they are capable of stimulating many cells including neutrophils (4–6). Phorbol esters have been shown to bind to specific cell receptors in order to exert their functions (7). Several studies indicate that intracellular calcium may be involved in the effect of phorbol esters (8). A recent study has shown that upon exposure to phorbol myristate acetate (PMA),[1] a significant proportion of total neutrophil calcium is extruded into the extracellular medium, suggesting stimulation of calcium extrusion mechanisms (9).

We have recently demonstrated that inside-out vesicles from the plasma membrane of PMA-treated guinea pig and human neutrophils can accumulate large amounts of calcium in a calmodulin sensitive, Mg-ATP-dependent process (10). The calcium accumulation has been attributed to a plasma membrane calcium pump with characteristics similar to that described in other tissues (11, 12).

An analysis of the effect of PMA on the plasma membrane calcium pumping activity and on the levels of free cytosolic calcium ($[Ca^{++}]_i$) in neutrophils forms the basis of this report.

Methods

Chemicals. Quin 2 AM was purchased from Lancaster Synthesis, England; Ionophore A23187 from Calbiochem-Behring Corp., American Hoechst

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1. *Abbreviations used in this paper:* 4α-phorbol, 4α,9α,12β,13α,20-pentahydroxytiglia-1-6-dien-3-one; 4β-phorbol, 4β,9α,12β,13α,20-pentahydroxytiglia-1-6-dien-3-one; $[Ca^{++}]_i$, free cytosolic calcium concentrations; FMLP, formyl-methionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate.

Corp., San Diego, CA; PMA, 4α,9α,12β,13α,20-pentahydroxytiglia-1-6-dien-3-one (4α-phorbol), 4β,9α,12β,13α,20-pentahydroxytiglia-1-6-dien-3-one (4β-phorbol), and formyl-methionyl-leucyl-phenylalanine (FMLP) were obtained from Sigma Chemical Co., St. Louis, MO.

ATP-dependent calcium uptake by plasma membrane-enriched fractions from guinea pig neutrophils. Preparation of plasma membrane-enriched fractions and measurement of ATP-dependent calcium transport was carried out as previously described (10).

In brief, neutrophils purified from guinea pig peritoneal exudates were incubated for 5 min at 37°C in a medium containing 130 mM NaCl, 4 mM KCl, 50 μM $CaCl_2$, and 10 mM sodium phosphate buffer, pH 7.4, in the absence or in the presence of PMA (1.6 μM). Plasma membrane-enriched fractions ("podosomes") were subsequently obtained by gentle sonication and differential centrifugation. Ca uptake by podosomes was measured in a medium containing 100 mM KCl; 30 mM imidazole-HCl, pH 7; 5 mM azide; 5 mM Mg-ATP; and various concentrations of $CaCl_2$ containing ^{45}Ca and EGTA by the Millipore filtration technique (Millipore Corp., Bedford, MA). Ca uptake rates were expressed as nanomoles Ca per milligram total protein per minute after the values bound in the absence of ATP were subtracted. In the absence of ATP, <0.5 nmol Ca/mg protein became associated with the membranes in <1 min and the values did not increase thereafter.

The free Ca concentration of solutions in the presence of ATP with or without EGTA were calculated by means of the computer program of Perrin and Sayce (10). The calculated values correlated well with direct measurements of free calcium performed with calcium electrodes, as described previously (10). Bovine brain calmodulin used in these experiments was obtained as described previously (13). The proportion of inside-out vesicles in the membrane preparation was assessed by the activity of 5'-nucleotidase, an ectoenzyme of guinea pig granulocytes, in the presence or absence of Triton X-100, as described previously (10).

Measure of cytosolic free calcium by the intracellular fluorescent Ca indicator Quin 2. The isolated guinea pig neutrophils were resuspended in a medium containing 138 mM NaCl, 6 mM KCl, 1.2 mM P_i, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 5.6 mM glucose, 5 mM $NaHCO_3$, and 20 mM Hepes (pH 7.4 at 37°C), supplemented with 10% autologous serum, until used. Quin 2 loading was performed as described previously (14). The calibration of Quin 2 fluorescence as a function of cytosolic free Ca^{++} was described previously (14). Excitation and emission wavelengths were 339±6 and 492±5 nm, respectively.

Results

Fig. 1 *A* shows that when plasma membrane vesicles are isolated from guinea pig neutrophils previously treated for 5 min with PMA, the rate of ATP-dependent Ca accumulation is more than doubled compared with vesicles prepared without PMA. Addition of the calcium ionophore A23187 released ^{45}Ca in the supernatant from both PMA and control vesicles to levels obtained in the absence of Mg-ATP, indicating that calcium had been accumulated by both vesicle preparations against a concentration gradient.

No difference in ^{45}Ca associated with both membrane preparations was seen in the absence of ATP, indicating that PMA does not change calcium binding to the plasma membrane. The specificity of the stimulatory effect by PMA on the calcium pump in the plasma membrane was studied by incubating guinea pig neutrophils with an inactive phorbol analogue, 4β-phorbol. 4β-phorbol (1.6 μM) slightly inhibited active calcium transport when compared with vesicles prepared from neutrophils in-

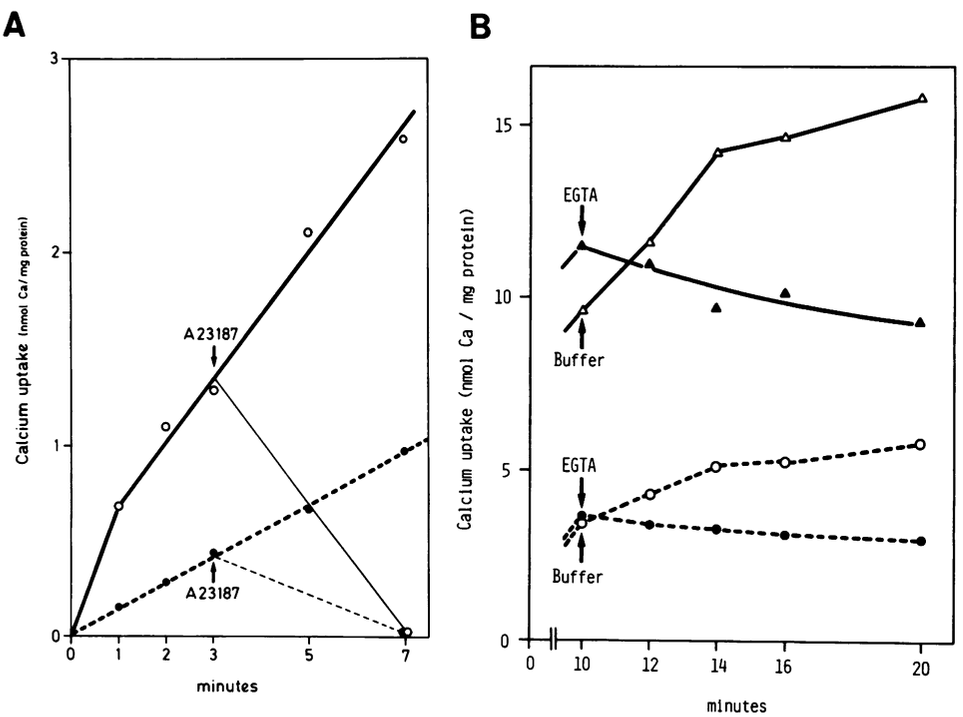

Figure. 1. (*A*) Calcium uptake by plasma-enriched fractions from guinea pig neutrophils that were incubated in the presence (— ○ —) or in the absence (- - - ● - - -) of PMA. Ionophore A23187 (5 μM) was added at 3 min. (*B*) Effect on calcium fluxes by the lowering of free Ca^{++} in the incubation medium. Calcium uptake was performed over 10 min, as described in *A*, by membranes prepared in the same way (——, with PMA; -----, without PMA). After 10 min of uptake, an aliquot was removed from the incubation medium for ^{45}Ca determination and either EGTA (▲, ●) or the same volume of buffer (△, ○) was added. Ca uptake was also determined at 12, 14, 16, and 20 min of experimentation. The addition of EGTA lowered free Ca in the incubation medium from pCa 4.9 to pCa 7.6.

cubated in the absence of a phorbol ester (25±10% inhibition, mean±SD, n = 4).

Two lines of evidence indicate that PMA pretreatment specifically increases the calcium pumping activity rather than stimulating calcium accumulation by indirect effects: (*a*) The proportion of inside-out vesicles, as assessed by 5'-nucleotidase activity, an ectoenzyme of the guinea pig granulocyte, in the presence or absence of Triton X-100, is similar for control vesicles and vesicles from PMA-treated neutrophils, i.e., 45±8% and 50±5%, respectively (mean±SD, n = 9). (*b*) No difference in the passive Ca permeability between the two population of vesicles is observed: Fig. 1 *B* shows that the addition of EGTA, when Ca uptake is almost completed, induces a slow release of ^{45}Ca into the supernatant; the kinetics of release is similar in controls and PMA-pretreated vesicles. In both preparations, the percentage of Ca released in the 10 min following EGTA addition is ~20% of the uptake during the first 10 min of the experiment.

Fig. 2 *A* shows that the stimulation of Ca accumulation by the vesicles obtained from PMA-treated neutrophils requires at least 1 or 2 min of preincubation of the phorbol ester with intact cells and it is maximal after about 5 min. The lag phase of this activation is similar to the latency of PMA-induced superoxide production in intact cells (15). Fig. 2 *B* shows that preincubation with PMA increases the maximum velocity (V_{max}) but not the Michalis constant (K_m) of Ca transport. The K_m of Ca^{++} of calcium transport by vesicles prepared in the presence of PMA was 0.38±0.12 μM Ca^{++}, whereas the K_m of Ca^{++} of those vesicles prepared simultaneously in the absence of this phorbol ester was 0.43±0.04 μM Ca^{++} (n = 3, mean±SD). This effect is different from the stimulatory effects of calmodulin on plasma membrane Ca-ATPases, which affects both the K_m and the V_{max} of Ca transport (11). Furthermore, addition of exogenous calmodulin (10 μg/ml) to control and PMA-pretreated vesicles causes no further increase of Ca accumulation, indicating that enough calmodulin remains bound to the Ca pump units.

When the experimental conditions are reversed, i.e., when PMA (1.6 μM) is added at 37°C for 5 min to plasma membrane vesicles isolated from untreated cells, no subsequent activation of the rate of calcium accumulation is observed. These results suggest that PMA activation requires some component(s) present in intact cells, which are lost during vesicle preparation. However, as shown by our results, the effect of PMA on Ca uptake withstood the manipulations required to isolate the vesicles and persisted for several hours after their isolation.

Assessment of free cytosolic Ca with the intracellularly trapped fluorescent Ca indicator Quin 2 allowed us to study the effect of PMA added to intact guinea pig and human neutrophils. Upon addition of PMA (100 nM), cytosolic free Ca did not rise, but decreased slightly below the resting level after a lag time <1 min (Fig. 3 *C*). Fig. 3 *C* shows also that PMA prevented almost completely the cytosolic free Ca rise induced by the chemotactic peptide, FMLP. This effect was noted even 15 min after the addition of PMA (not tested thereafter). In the absence of PMA, FMLP induced a rise of cytosolic free Ca to

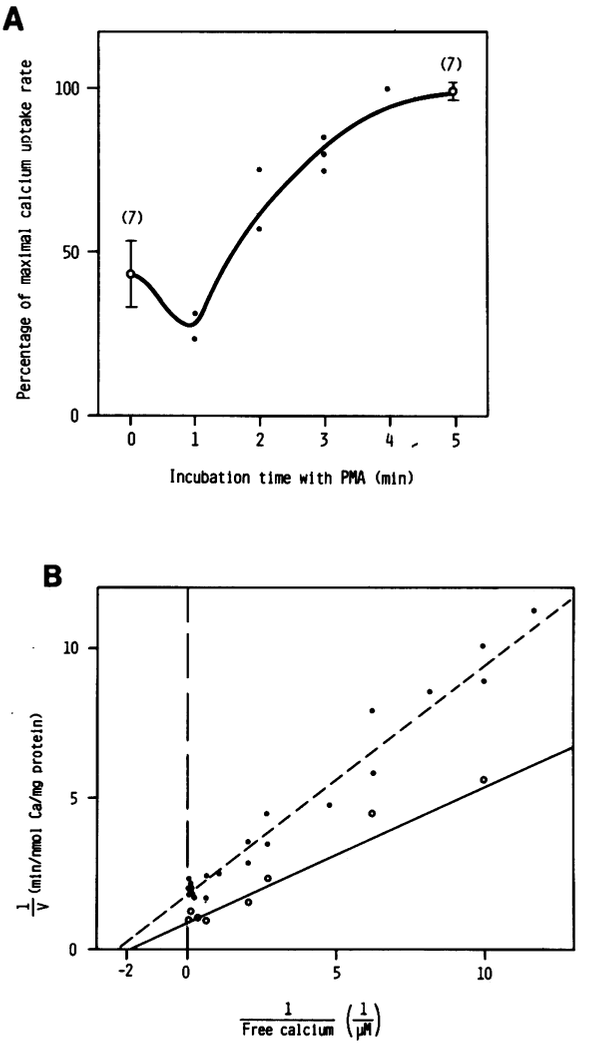

Figure 2. (*A*) Time course of PMA activation. Neutrophils were incubated with PMA (1.6 μM) for various durations of time; plasma membrane-enriched fractions were obtained and tested for initial Ca uptake at 3 min after addition of Mg-ATP. At 0 and 5 min incubation, mean and SD (bars) of seven experiments are shown. (*B*) Effect of PMA on the K_m and V_{max} of Calcium transport. As plotted from this graph, membranes prepared without PMA (- - - ● - - -) had a K_m for Ca of 430 nM and V_{max} of 0.55 nmol Ca/mg protein per min, and the membranes prepared simultaneously with PMA (1.6 μM) (— o —) exhibited a K_m for Ca of 518 nM and V_{max} of 1.05 nmol Ca/mg protein per min.

800 nM in the same batch of Quin 2 loaded cells (Fig. 3 *A*). Moreover, PMA added after FMLP accelerated the return of cytosolic free Ca to basal levels (Fig. 3 *B*). Qualitatively similar tracings were obtained in neutrophils from a patient with chronic granulomatous disease and in guinea pig peritoneal exudate neutrophils. At 10 nM, PMA added 3 min before the addition

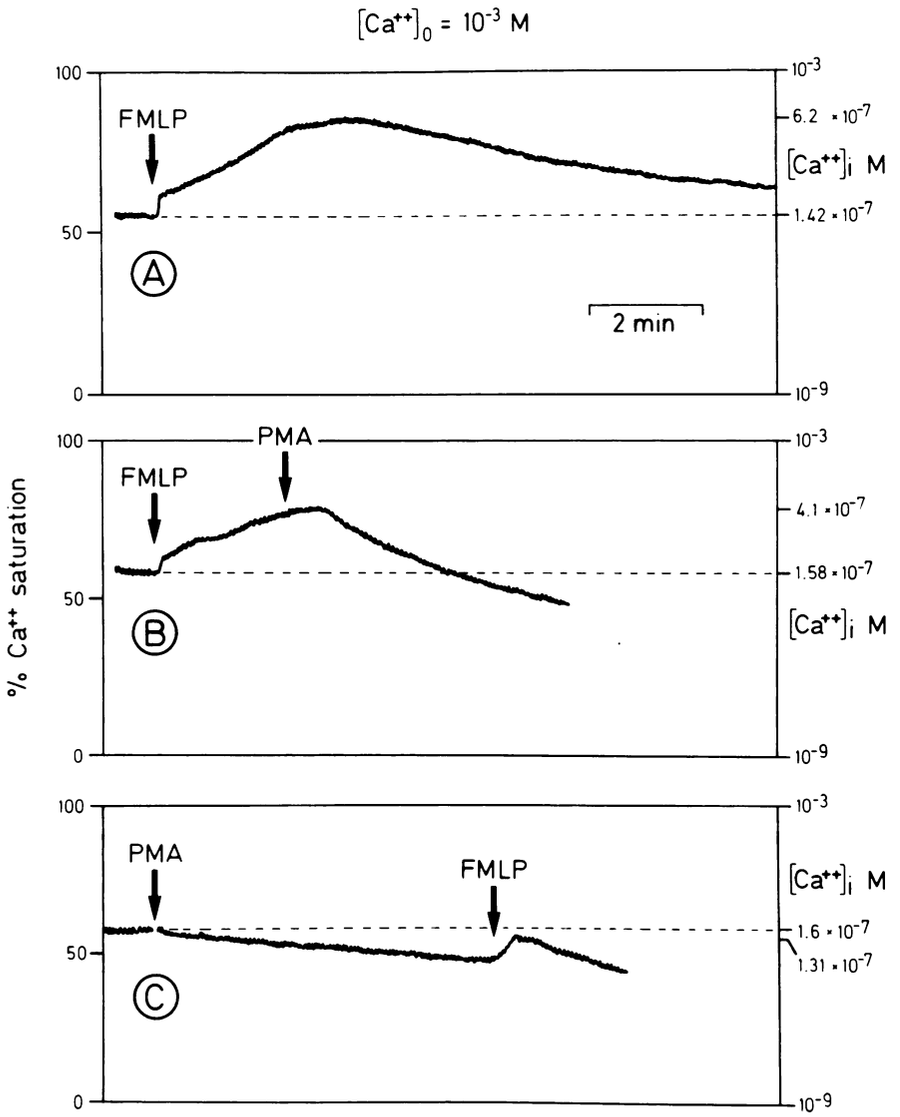

Figure 3. Effect of PMA and FMLP on the levels of cytosolic free Ca in human neutrophils, loaded with Quin 2. PMA = 100 nM, FMLP = 0.1 μM. Experiments performed in the same batch of cells (Quin 2 loading: 1.2 nmol/10^6 PMN).

of FMLP (2×10^{-7} M) significantly increased the return of cytosolic free calcium to basal levels induced by the chemotactic peptides (4.5±1.2 min instead of 8±1.5 min for a batch of human neutrophils loaded with 0.6 nmol Quin 2/10^6 PMN; mean±SD of $n = 5$, $P < 0.02$ by paired t test). By contrast, no effect of the FMLP-induced changes in free cytosolic calcium was observed when human neutrophils were preincubated with two inactive phorbol analogues, 4α-phorbol (100 nM) or 4β-phorbol (100 nM).

Discussion

Three main mechanisms may explain the stimulatory effect of PMA on the ATP-dependent Ca pumping activity present in the plasma membrane of neutrophils: PMA pretreatment may increase the number of Ca pump units in the plasma membranes; PMA could modify qualitatively the plasma membrane Ca pump units and thereby increase their V_{max}; and PMA could alter the membrane lipid structures surrounding the pump units. As far as the first mechanism is concerned, one could postulate that PMA induces a recruitment of previously inactive Ca pump units in the plasma membrane, or modifies the recycling of the Ca pump monomers from the membrane to intracellular vesicles and vice-versa. In support of this, PMA is known to induce exocytosis of secondary granules in neutrophils (16); although the presence of calcium pumps in the primary and secondary granules has not been demonstrated yet, such a location would lead to an increase of the number of Ca pump units per surface

area after their fusion with the plasma membrane. The second possibility is more likely, since it is consistent with the more general effect of phorbol esters on calcium homeostasis in a number of cell types including lymphocytes, which possess a very limited amount of intracellular vesicles (17). One likely candidate reaction for an enzymatic modification of neutrophil Ca-ATPase is its direct or indirect phosphorylation by protein kinases activated by PMA. This hypothesis is in agreement with at least three well-documented facts: (*a*) phosphorylation of phospholamban is known to activate the Ca pump of sarcoplasmic reticulum and sarcolemma by cAMP-dependent kinases (18, 19); (*b*) phorbol esters are known to activate protein c kinase in vitro, and phorbol ester receptors are copurified with protein c kinase (20); and (*c*) phosphorylation of four major bands are known to be an early event in the interaction of PMA with intact neutrophils (21).

As a third possibility, an effect of PMA on lipid fluidity has been suggested on the basis of its effects on 1,6-diphenyl-1,3,5-hexatriene fluorescence (22). Though the results obtained with this probe are difficult to interpret in systems as complicated as intact cells, an effect of PMA on lipid domains surrounding the plasma membrane calcium pump cannot be excluded.

Whatever the mechanism of PMA stimulation of calcium pumping activity, this activation results in a small decrease of resting free Ca^{++} and inhibition of the $[Ca^{++}]_i$ rise induced by chemotactic peptides. It is important to note that similar results on the effect of phorbol esters on the levels of cytosolic free calcium using Quin 2 were obtained previously in lymphocytes by Tsien et al. (17), indicating that the effect of phorbol esters on calcium pumps is a widespread phenomenum.

Besides demonstrating that one of the earliest physiological events elicited by PMA on neutrophils is the stimulation of Ca extrusion via the plasma membrane Ca pump, our studies suggest that this occurs by a Ca-independent pathway because it is not preceded by a rise in cytosolic free calcium, nor it is mediated by an alteration in the affinity constant of the pump for Ca.

Recent data would suggest that diacylglycerol produced by stimulus-dependent breakdown of phosphoinisitide is involved in this calcium-independent activation (23).

It is generally believed that Ca pump stimulation occurs if cytosolic free calcium is increased because more substrate is available to the pump, and possibly, because of secondary increased calmodulin binding (23). However, the stimulation of the calcium pump by increased $[Ca^{++}]_i$ is a reversible phenomenon and the activity returns to basal levels when $[Ca^{++}]_i$ returns to resting values. On the contrary, PMA stimulation seems to be essentially irreversible in intact cells and in isolated vesicles. Overall, these results suggest that the plasma membrane calcium pump may be activated by various different mechanisms. The demonstrated stimulation of the Ca pumping activity of the plasma membrane provides a simple mechanism to explain the effects of PMA on Ca homeostasis in various cell systems. Whether this activation of Ca extrusion is a critical factor in the stimulation of cell functions remains to be established.

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