Regulation of Erythrocyte Ca\textsuperscript{2+} Pump Activity by Protein Kinase C*

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The activity of the plasma membrane Ca\textsuperscript{2+} pump is of critical importance to the maintenance of cellular Ca\textsuperscript{2+} homeostasis (1–5). This pump catalyzes the ATP-dependent exchange of internal Ca\textsuperscript{2+} for external H\textsuperscript{+} (1) and is responsible for the maintenance of a 5,000–10,000-fold Ca\textsuperscript{2+} concentration gradient across the plasma membrane. A major mechanism by which hormones and neurotransmitters regulate cell function is by altering Ca\textsuperscript{2+} influx rates across the plasma membrane of target cells (1, 6–8). In nearly all instances in which an extracellular messenger induces a sustained increase in Ca\textsuperscript{2+} influx rate, there is a compensatory increase in Ca\textsuperscript{2+} efflux rate so that during the sustained response, there is an increase in Ca\textsuperscript{2+} cycling across the plasma membrane without a net accumulation of Ca\textsuperscript{2+} by the cell (6, 8). The biochemical and cellular basis for the homeostatic regulation of Ca\textsuperscript{2+} pump activity is not completely known, but one very important feature is the direct allosteric activation of the pump by calmodulin (9–11). A rise in intracellular Ca\textsuperscript{2+} concentration leads to Ca\textsuperscript{2+}-dependent association of CaM' with the pump. As a consequence, the \(V_{max}\) increases and the \(K_m\) for Ca\textsuperscript{2+} decreases (12).

In recent years, a second major pathway by which Ca\textsuperscript{2+} regulates cell function has been recognized: the protein kinase C pathway (13, 14). There is evidence in several different cellular systems that the activity of the plasma membrane-associated protein kinase C is controlled by the rate of Ca\textsuperscript{2+} influx across this membrane (6, 8). Also, there is evidence which suggests that phorbol ester activators of protein kinase C stimulate Ca\textsuperscript{2+} efflux from certain cells (15–21) and in high concentrations stimulate the ATP-dependent uptake of Ca\textsuperscript{2+} into inside-out vesicles prepared from neutrophils (22). However, there is no direct evidence that under physiologic circumstances protein kinase C is an activator of the plasma membrane Ca\textsuperscript{2+} pump itself.

The present experiments were undertaken to determine whether or not protein kinase C has any effect upon Ca\textsuperscript{2+} pump activity. The systems chosen for this examination were IOV prepared from human erythrocytes and purified Ca\textsuperscript{2+}-ATPase from the same source. This erythrocyte system was chosen because it is the most extensively studied of the plasma membrane Ca\textsuperscript{2+}-ATPases (2, 12) and has been traditionally viewed as a model for other plasmalemma calcium pumps. Furthermore, the human erythrocyte has been shown to possess protein kinase C (23), and this enzyme binds to red cell IOV in a Ca\textsuperscript{2+}- and diacylglycerol (or phorbol ester)-dependent manner (24).

MATERIALS AND METHODS

The \[^{45}\text{Ca}\text{Cl}_2\] and \[^{32}\text{P}\text{ATP were obtained from Amersham Corp. DAEA-cellulose, phenyl-Sepharose, cyanogen bromide-activated Sepharose 4B, TPA, histone, and calmodulin were purchased from Sigma. Fractogel HW55S was obtained from Rainin Instrument Co. Inc. and AH-Sepharose from Pharmacia LKB Biotechnology Inc. The 1,2-diolein was purchased from Avanti Polar Lipids. "Baker-flex" DAEA-cellulose TLC plates were obtained from J. T. Baker Chemical Co. Alkaline phosphatase (calf intestine, molecular biology grade) was purchased from Boehringer Mannheim. Coomassie Blue staining of SDS-polyacrylamide gel electrophoresis gels showed the phosphatase preparation to be composed of a single polypeptide of about 55,000 daltons.

The calmodulin used for purification of the Ca\textsuperscript{2+}-ATPase was prepared from bovine brain according to the method of Gopalakrishna and Anderson (25). SDS-polyacrylamide gel electrophoresis analysis of the final calmodulin preparation showed it to be absolutely pure as judged by Coomassie Blue staining.

The abbreviations used are: CaM, calmodulin; IOV, inside-out vesicles; protein kinase C, CaP\textsuperscript{2+}/phospholipid-dependent enzyme; SDS, sodium dodecyl sulfate; TPA, 12-O-tetradecanoylphorbol-13-acetate; TSE, tris (hydroxymethyl)aminomethane; PC, phosphatidylcholine; EGTA, ethyleneglycolbis(oxyethylenedinitriolo)tetraacetic acid; PIPES, 1,4-piperazine-dithanesulfonic acid. HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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Purification of Protein Kinase C—Protein kinase C was purified from bovine brain by modifications of the procedure of Kikkawa et al. (26). All steps were performed at 4°C. Freshly excised tissue was immediately homogenized by a Polytron apparatus in 20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 50 μM leupeptin, and the homogenate was spun at 100,000 × g for 25 min. The supernatant was added to 250 ml of DRAE-cellulose preequilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol). The mixture was stirred for 1 h; the resin was filtered on a scinttered glass funnel and washed with 8 volumes of buffer A and 8 volumes of buffer B containing 20 mM NaCl. Enzyme was then eluted with 3 volumes of buffer A, 90 mM NaCl.

The eluent was stirred for 1 h with 60 ml of threonine-Sepharose suspended in buffer A. (Threonine was coupled to AH-Sepharose by the method of Kikkawa et al. (26)). The resin was then filtered on a scinttered glass funnel and washed with 6–7 volumes of buffer A and 4 volumes of buffer B, 400 mM NaCl. Protein kinase C activity was then eluted with 250 ml of buffer B, 800 mM NaCl.

This eluent was adjusted to 1.5 M NaCl and applied to a 10-ml phenyl-Sepharose column. This column was washed with 5 volumes of buffer C and 5 volumes of buffer A, followed by 50 ml of buffer A.

Pooled kinase activity was concentrated by overnight dialysis against a solution of 7.5% polyethylene glycol in buffer A. The final step of purification was gel filtration on a Sephadex G-25 column with buffer A, 100 mM NaCl. Pooled protein kinase C was stored at −80°C in buffer A, 100 mM NaCl, 10% glycerol (v/v), and 0.05% Triton X-100. The enzyme remained active for more than 6 months.

Assay of Protein Kinase C—Kinase activity was assayed by Ca2+/phospholipid-dependent phosphorylation of histone 1 (Sigma histone fraction IIIS). Ten microliters of enzyme was assayed in buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1.2 mM CaCl2 (free Ca2+, 200 μM), 20 μg/ml phosphatidyserine (PS), 2 μg/ml dolinie, and 200 μg/ml histone in a total volume of 0.1 ml. PS and dolinie, dissolved in chloroform and dried under nitrogen, were suspended by sonication; the solutions were prewarmed for 1 min at 30°C, and 0.5 mM MgCl2 was added to initiate the reaction. After 5 min at 30°C, the reaction was stopped with 0.8 ml of ice-cold 20% trichloroacetic acid. Samples were filtered on 0.45-μm Millipore filters and were washed with 5 ml of 5% trichloroacetic acid. Filters were counted in H2O in a scintillation counter. Activity in the presence of Ca2+ alone was subtracted from that obtained with Ca2+/PS and PS alone. One unit of protein kinase C activity was defined as that amount transferring 1 pmol of phosphate to histone per min under these conditions.

Preparation of IOV. Inside-out vesicles were prepared from freshly frozen bovine blood with some modification of the procedure described elsewhere (5). To rid the membranes of endogenous calmodulin and protein kinase C, intact cells were washed three times in 2 volumes of 155 mM NaCl, 5 mM sodium phosphate, pH 7.4 (phosphate-buffered saline), containing 10 mM EGTA. Cells were then lysed in 12 volumes of 5 mM sodium phosphate, pH 8.2, 10 mM EGTA. Membranes were washed three times in the same phosphate buffer with decreasing amounts of EGTA: 5, 1, and 0 mM. They were allowed to endovesiculate for 1 h in 0.5 mM sodium phosphate, pH 8.2, and all further steps were as outlined in Ref. 5. Dextran was used at 0.96 g/ml. All steps were performed at 0–4°C. Final suspensions of IOV were rapidly frozen in liquid nitrogen and stored at −80°C. They were slowly thawed at room temperature as needed and were used within 3 weeks of preparation.

The percentage of vesicles obtained in the inside-out orientation was calculated by the Calcon computer program kindly provided by Dr. J. S. Tash of the Department of Cell Biology, Baylor University, Houston, Texas.

Purified protein kinase C was added to 40–370 units/ml (total volume, 300–500 μl). TPA dissolved in ethanol was added to vesicles at 0–100 nM (final concentration of ethanol ≤1%). Dioxane, dissolved in chloroform, was dried under nitrogen and resuspended in ethanol; it was added to a final concentration of 10–100 μg/ml. Equivalent volumes of protein kinase C storage buffer and ethanol were added to controls as appropriate.

Vesicles were prewarmed at 37°C for 2 min, and ATP (vanadate-free) was added to 0.9 mM to initiate transport. At appropriate time points, 50-μl aliquots were vacuum-filtered on 0.45-μm Millipore filters presoaked in 250 mM sucrose, 40 mM NaPipes, pH 7.2. Filters were washed with 6 ml of ice-cold 250 mM sucrose, 40 mM NaPipes. Uptake of Ca2+ by IOV was then determined by liquid scintillation spectrometry.

Purification of Erythrocyte Ca2+/ATPase—A modification of the procedure developed by Carafoli and co-workers (10, 28–30) was used. Recently outdated red blood cells from the blood bank (150 ml packed volume) were washed twice in 150 mM NaCl, 5 mM sodium phosphate, pH 7.4 (phosphate-buffered saline), containing 10 mM EGTA. They were then eluted in 2 volumes of 5 mM sodium phosphate, pH 8.2 (5P8), 10 mM EGTA as for preparation of IOV. Membranes were washed three times in 5P8 without EGTA to rid them of soluble proteins, including hemoglobin, and then once more in 5P8, 10 mM EGTA to deplete them of bound calmodulin. Finally, membranes were washed three times in 10 mM HEPES, pH 7.5, 130 mM NaCl, 0.5 mM MgCl2, 5 mM 2-mercaptoethanol (HEPES buffer). CaCl2 to 50 μM was included in the last wash (HEPES/Ca2+ buffer).

To extract the Ca2+-ATPase, packed membranes were resuspended in HEPES/Ca2+ buffer in a total volume of 30 ml. Triton X-100 was added to 0.1% (v/v). The solution was incubated on ice for 15 min. After centrifugation at 40,000 × g for 6 min, the supernatant was aspirated and membranes were reextracted in the same manner. PS was added to the combined supernatants to stabilize the ATPase (final concentration, 0.4 mg/ml PS in 94 ml).

The membrane extract was applied to a 0.7-ml calmodulin-Sepharose affinity column, preequilibrated in HEPES/Ca2+ buffer with 0.4 mg/ml PS, 0.4% Triton X-100. (Purified calmodulin was coupled to cyanogen bromide-activated Sepharose 4B as described in Ref. 10.) The column was washed with 10 volumes of HEPES/Ca2+ buffer with 0.4 mg/ml PS, 0.4% Triton X-100, 10 mM EGTA was added to elute Ca2+ dependent transport. The solution was incubated with CaM, followed by 5P8 buffer. The column was washed with 1 mM EGTA in HEPES buffer, 0.4 mg/ml PS, 0.05% Triton X-100.
Protein Kinase C Regulation of the Ca\(^{2+}\) Pump

**FIG. 1.** Ca\(^{2+}\) uptake by IOV pretreated with alkaline phosphatase. The accumulation of Ca\(^{2+}\) by IOV was measured as a function of time. The experiments were conducted at 100 \(\mu\)M free Ca\(^{2+}\) (EGTA was present at 1 mM). ●, incubation of IOV with no added activators; □, incubation with 147 units (370 units/ml) of protein kinase C (PKC) and 25 nM TPA. Results from eight separate experiments are shown. If either protein kinase C alone or TPA alone were present, the uptake was not different from basal. UACE, units of acetylcholinesterase activity.

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**FIG. 2.** A and B, effect of protein kinase C on Ca\(^{2+}\) transport in alkaline phosphatase-pretreated IOV; C and D, effect of TPA on Ca\(^{2+}\) transport in alkaline phosphatase-pretreated IOV. A, time course of ATP-dependent Ca\(^{2+}\) uptake by IOV at 0.6 \(\mu\)M free Ca\(^{2+}\), with 100 \(\mu\)M TPA present in all samples. IOV were incubated with no added protein kinase C (PKC) (nl, ▲) or with various doses of purified protein kinase C as indicated (15–147 units (u) of kinase activity/sample (○)). HT, □, incubation with 147 units of heat-treated protein kinase C. After 40 min of Ca\(^{2+}\) uptake, 5 \(\mu\)M A23187 was added to release accumulated Ca\(^{2+}\). Retained Ca\(^{2+}\) was measured 15 min later. ATP-independent binding of Ca\(^{2+}\) by IOV was subtracted from all points. UACE, units of acetylcholinesterase activity. B, stimulated Ca\(^{2+}\) uptake rates (during the linear, post-lag phase) plotted as a function of added protein kinase C. The ordinate represents the -fold increase over the rate measured in the absence of protein kinase C. C, time course of ATP-dependent Ca\(^{2+}\) uptake conducted at 0.6 \(\mu\)M free Ca\(^{2+}\) and varying concentrations of TPA. IOV were present at the same concentration as above, and 147 units of protein kinase C (370 units/ml) were added to all samples. ▲, no added TPA; ○, TPA added in amounts indicated. A23187 was added as described above. Before addition to the Ca\(^{2+}\) uptake incubation medium, these IOV were stored on ice for approximately 6 h. D, stimulated rates of Ca\(^{2+}\) uptake plotted against the added concentration of TPA. The ordinate represents -fold stimulation of the rate measured in the absence of TPA. Half-maximal stimulation was found at approximately 10 nM TPA.

Collected fractions were assayed for ATPase activity as described below. The Calcon program was used to calculate amounts of EGTA and Ca\(^{2+}\) needed to obtain desired free Ca\(^{2+}\) concentrations. Peaks of activity were pooled, 10% glycerol (v/v) was added, and enzyme was dialyzed by micro-flow dialysis against HEPES buffer, 0.4 mg/ml PC, 0.05% Triton X-100, 10% glycerol, and 50 \(\mu\)M free Ca\(^{2+}\) (EGTA, 9.1 mM total). Ca\(^{2+}\) and glycerol were included to stabilize the activity during dialysis and storage. The ATPase was frozen and stored at -80 °C.

**ATPase Assay—**For the purified Ca\(^{2+}\)-ATPase, 10–30 \(\mu\)l of enzyme were incubated at 37 °C in 40 mM NaPIPES, pH 7.2, 5 mM MgPIPES, 5 mM sodium phosphate, 100 \(\mu\)M free Ca\(^{2+}\), and 4.2 \(\mu\)M A23187 to avoid possible sequestering of Ca\(^{2+}\) in lipid micelles (total volume, 60 \(\mu\)l). Calmodulin, protein kinase C/phosphatidylycerine, TPA, or diol-ein were added to assay mixtures as needed. The reaction was started with addition of \[^{32}\]P]ATP, 100 \(\mu\)M. At various time points (15–25 min), 15–\(\mu\)l aliquots were withdrawn, and the reaction was stopped by addition of SDS and EDTA to 2% and 8 mM, respectively. One-microliter samples were spotted in triplicate on DEAE-cellulose TLC plates (20 x 5 cm). The plates were developed in 0.1 M sodium acetate buffer, pH 4.75, and then exposed to Kodak X-AR5 x-ray film for 2-3 h for autoradiography. Plates were then cut; \[^{32}\]P]ATP and \(^{38}\)F, spots were each counted in H,O. Rates of ATP hydrolysis in the presence of Mg\(^{2+}\) and 5 mM EGTA were subtracted to obtain Ca\(^{2+}\)-dependent rates.
RESULTS

The results of time course experiments performed on alkaline phosphatase pretreated vesicles are shown in Fig. 1. The data represent the combined results from eight separate experiments performed on three different IOV preparations, after 1–15 days of storage at −80 °C. At 100 μM free Ca²⁺, the mean basal Ca²⁺ uptake rate with alkaline phosphatase-treated IOV was 1.92 nmol/min/IOV units of acetylcholinesterase activity (range 1.5–2.4), and uptake was always linear for at least 40 min. In the presence of maximal protein kinase C and 25 nM TPA, the mean initial Ca²⁺ transport rate was 5.26 nmol/min/IOV units of acetylcholinesterase activity (range 4.7–5.5), representing approximately a 2.7-fold stimulation. Uptake of Ca²⁺ under these conditions was not linear after 20 min.

Dose Dependence of Protein Kinase C Stimulation—The effect of various doses of protein kinase C upon time courses of IOV Ca²⁺ uptake is shown in Fig. 2A. TPA was held constant at 100 nM. This experiment was performed at 0.6 μM free Ca²⁺, which represents an intermediate value in the physiological range of intracellular free Ca²⁺ levels (about 0.05–2 μM). At 0.6 μM free Ca²⁺, a brief delay in the protein kinase C-induced stimulation was noted, and this lag time was also dependent upon the dose of kinase added. Initial stimulated rates of Ca²⁺ uptake were calculated at these and other doses of protein kinase C, and the dose-response relationship is shown in Fig. 2B. The rate of Ca²⁺ transport in the presence of TPA but no added protein kinase C was indistinguishable from the basal rate (no additions).

When protein kinase C was heat-treated prior to addition (70 °C, 10 min), a previously maximal dose was completely ineffective in stimulating Ca²⁺ transport (Fig. 2A). Because calmodulin is heat stable, this result eliminates the possibility that the observed stimulation might be due to calmodulin contamination in the kinase preparation.

In all cases, addition of ionophore A23187 at 40 min released Ca²⁺ to approximately the same basal levels. Hence, the measured increases in Ca²⁺ accumulation resulted from increased transport across the membrane and not simply from enhanced binding of Ca²⁺ to the vesicles.

TPA Dependence of Protein Kinase C Stimulation—Protein kinase C stimulation of Ca²⁺ uptake was dependent upon the dose of phorbol ester (TPA) added, as shown by the time courses in Fig. 2C. A delay in activation was observed in these experiments as well. At 0.6 μM free Ca²⁺ and at 147 units (=370 units/ml) of protein kinase C, half-maximal activation of transport rate occurred at approximately 10 nM TPA (Fig. 2D), which is consistent with other reports of its potency as an activator of protein kinase C (31–33).

Initial stimulated Ca²⁺ transport rates in the presence of maximal protein kinase C and phorbol ester were quite similar as measured in several different experiments. However, a late phase deactivation of Ca²⁺ uptake often occurred. This deactivation was not observed with IOV in the absence of protein kinase C and TPA. The timing and extent of this inhibition was quite variable; occasionally it was not observed at all within the time course studied (compare Fig. 2, A and C). Storage of vesicles on ice for several hours after phosphatase pretreatment reduced or eliminated the late phase deactivation.

Diacylglycerol as a Protein Kinase C Activator—The diacylglycerol, 1,2-diolein, was as effective as TPA in supporting protein kinase C stimulation of Ca²⁺ uptake at 0.6 μM free Ca²⁺ (Fig. 3). Maximal stimulation at this Ca²⁺ concentration was obtained with 20 μg/ml diolein. As with TPA, diolein did not stimulate Ca²⁺ transport in the absence of added protein kinase C. Eventual inhibition of the protein kinase C stimulatory effect was observed with diolein as with TPA.

Reversal of Protein Kinase C Stimulation with Alkaline Phosphatase—To determine whether activation of Ca²⁺ uptake by protein kinase C could be reversed by phosphatase treatment, alkaline phosphatase-pretreated IOV were incubated at 37 °C for 5 min with 10 μM free Ca²⁺ and 0.5 mM ATP, in the presence of maximal protein kinase C and TPA. Control IOV were similarly incubated, but with no protein kinase C or TPA added. All vesicles were then centrifuged and washed, and ATP-dependent Ca²⁺ uptake was measured at 0.6 μM free Ca²⁺ on a portion of both protein kinase C-stimulated and control IOV. The remainder of both samples was then split into 2 aliquots which were incubated for 10 min at

FIG. 3. Effect of 1,2-diolein on Ca²⁺ transport in alkaline phosphatase-pretreated IOV. A time course experiment was conducted at 0.6 μM free Ca²⁺ in the presence or absence of diolein and the presence or absence of protein kinase C (PKC), 147 units (370 units/ml). ○, Ca²⁺ uptake in the presence of protein kinase C and the indicated amounts of diolein; □, Δ, Ca²⁺ uptake in the absence of protein kinase C and diolein; or ●, in the presence of 50 μg/ml diolein but no added protein kinase C. The dotted line represents Ca²⁺ uptake in the presence of protein kinase C and 100 nM TPA ( ■). The values for 20, 50, and 100 μg/ml diolein in the presence of protein kinase C were not significantly different and are shown as the mean ± S.D. (n = 1 for each concentration of diolein). wACE, units of acetylcholinesterase activity.

FIG. 4. Effect of alkaline phosphatase on protein kinase C-stimulated IOV. All uptake experiments were conducted at 0.6 μM free Ca²⁺, as in Figs. 2 and 3. ○, IOV activated with maximal protein kinase C/TPA; O, protein kinase C-stimulated IOV incubated for 10 min at 37 °C, pH 7.7, with no alkaline phosphatase; ●, protein kinase C-stimulated IOV incubated under the same conditions with 70 units/ml alkaline phosphatase. △, control IOV incubated for 10 min at 37 °C, pH 7.7, with no alkaline phosphatase; ▲, control IOV incubated with alkaline phosphatase. Phosphatase was removed from all samples before initiation of Ca²⁺ uptake. wACE, units of acetylcholinesterase activity.
were measured. No ATP was present. Then, the resulting Ca\textsuperscript{2+} transport rates were measured.

As shown in Fig. 4, incubation of protein kinase C-stimulated IOV without alkaline phosphatase led to a decrease in the protein kinase C-stimulated Ca\textsuperscript{2+} uptake rate when ATP and 0.6 μM free Ca\textsuperscript{2+} were reintroduced. However, incubation with alkaline phosphatase further reduced the uptake rate to a value indistinguishable from that of control IOV that had not been preactivated with protein kinase C/TPA. Ca\textsuperscript{2+} uptake by control IOV before the 10-min, pH 7.7 incubation did not differ from that shown for control IOV after such treatment.

Ca\textsuperscript{2+} Dependence of Protein Kinase C Stimulation—The effects of protein kinase C and TPA on Ca\textsuperscript{2+} uptake rates were examined over a wide Ca\textsuperscript{2+} concentration range. IOV samples were incubated with Ca\textsuperscript{2+}, with or without maximal protein kinase C/TPA. Aliquots were filtered 15 min after addition of ATP, and Ca\textsuperscript{2+} binding in the absence of ATP was subtracted from each determination.

As shown in Fig. 5A, IOV in the absence of protein kinase C exhibited two phases of Ca\textsuperscript{2+} transport activity. A high affinity but low velocity phase was observed up to 10 μM free Ca\textsuperscript{2+}, and a second phase of low affinity but higher velocity was observed above 10 μM Ca\textsuperscript{2+}. These two Ca\textsuperscript{2+} "transport sites" have been reported previously, although the estimates of $K_m$ and $V_{max}$ in each phase have varied (12, 34).

Protein kinase C in the presence of TPA significantly increased initial Ca\textsuperscript{2+} transport rates above 0.1 μM free Ca\textsuperscript{2+}. Relative to basal rates in the absence of kinase, the greatest effect of protein kinase C was found at 2–5 μM Ca\textsuperscript{2+}. Thereafter, stimulated transport rates increased roughly in parallel with basal rates, indicating that the activating effect of protein kinase C was primarily upon the low velocity, high affinity Ca\textsuperscript{2+} transport phase. In the absence of phorbol ester, protein kinase C did not stimulate Ca\textsuperscript{2+} transport at any concentration of Ca\textsuperscript{2+} tested.

Ca\textsuperscript{2+} Dependence of Calmodulin-stimulated Ca\textsuperscript{2+} Uptake—In a similar set of experiments, the effect of calmodulin upon Ca\textsuperscript{2+} transport rates in alkaline phosphatase pretreated IOV was examined. In the presence of 7.5 μg/ml CaM, stimulation of Ca\textsuperscript{2+} transport occurred above 50 nM free Ca\textsuperscript{2+}, reaching a maximum at 2–5 μM Ca\textsuperscript{2+} (Fig. 5B). At higher concentrations of free Ca\textsuperscript{2+}, uptake rates progressively declined. Some investigators have reported this phenomenon (27, 35, 36), while others have found no such inhibition (11, 37). Under these conditions, CaM was a more potent activator of the Ca\textsuperscript{2+} pump than was protein kinase C.

Addition of maximal protein kinase C/TPA to CaM-IOV further stimulated Ca\textsuperscript{2+} uptake at free Ca\textsuperscript{2+} concentrations above 0.2 μM. Since the effects of the two activators were approximately additive at maximally effective doses of each, CaM and protein kinase C appear to activate Ca\textsuperscript{2+} transport...
through different mechanisms. No inhibition of Ca\(^{2+}\) transport above 10 \(\mu M\) free Ca\(^{2+}\) was observed when protein kinase C/TPA was added in combination with CaM.

**Kinetic Effects of CaM and Protein Kinase C**—A detailed kinetic analysis of the effects of either CaM or protein kinase C on high affinity Ca\(^{2+}\) transport was examined. Lineweaver-Burk plots of initial ATP-dependent Ca\(^{2+}\) uptake rates within the range of 0.2–10 \(\mu M\) free Ca\(^{2+}\) are shown in Fig. 6. Normal and CaM-IOV data are derived from Fig. 5: all Ca\(^{2+}\) uptakes at all Ca\(^{2+}\) concentrations in such vesicles were previously found to be linear when alkaline phosphatase-treated IOV were used.

To ensure that the Ca\(^{2+}\)-dependence of protein kinase C-stimulated Ca\(^{2+}\) transport reflected only the activity of a fully activated Ca\(^{2+}\) pump, and did not also involve a Ca\(^{2+}\)-dependence in the activation of protein kinase C itself, IOV were preactivated by exposure to protein kinase C/TPA, ATP, and 10 \(\mu M\) unbuffered \(\text{Ca}^{2+}\) for 5 min at 37 \(^\circ\)C. The concentration of free \(\text{Ca}^{2+}\) was then adjusted as appropriate by addition of EGTA-buffered, radiolabeled \(\text{Ca}^{2+}\). Subsequent uptake of \(\text{Ca}^{2+}\) was found to be linear for at least 15 min at all concentrations of free \(\text{Ca}^{2+}\). Protein kinase C/TPA did not significantly alter the apparent \(K_m\) for Ca\(^{2+}\) of IOV Ca\(^{2+}\) transport (0.9 \(\pm\) 0.3 \(\mu M\) Ca\(^{2+}\) versus 1.2 \(\pm\) 0.4 \(\mu M\) Ca\(^{2+}\) for control IOV). However, the apparent \(V_{max}\) was increased from 0.6 to 3.3 nmol/min/IOV units of acetylcholinesterase activity by prior protein kinase C-dependent activation. In contrast, CaM not only increased \(V_{max}\) (derived to be 10.0 nmol/min/IOV units of acetylcholinesterase activity) but also significantly decreased the apparent \(K_m\) for Ca\(^{2+}\) to 0.30 \(\pm\) 0.05 \(\mu M\) free Ca\(^{2+}\).

**Effects of Protein Kinase C at Low Ca\(^{2+}\) Concentrations**—The results presented above suggest that at free Ca\(^{2+}\) concentrations below 0.1 \(\mu M\), protein kinase C in the presence of TPA does not significantly activate IOV Ca\(^{2+}\) transport. However, extended time courses were conducted to measure Ca\(^{2+}\) uptake at 80 nM Ca\(^{2+}\), a concentration approximating basal erythrocyte intracellular free Ca\(^{2+}\) (36). In fact, after a prolonged lag phase (20–35 min in different experiments), protein kinase C/TPA eventually stimulated Ca\(^{2+}\) uptake almost as much as calmodulin did (Fig. 7A). When protein kinase C/TPA and CaM were added together, Ca\(^{2+}\) transport was further stimulated. The enhanced rate of uptake remained constant for a more prolonged period than that observed in the presence of CaM alone.

A similar experiment using diolein instead of TPA to activate protein kinase C showed that this diacylglycerol did not support protein kinase C stimulation of Ca\(^{2+}\) transport at such low Ca\(^{2+}\) concentrations (Fig. 7B). However, in another experiment, IOV were first exposed to protein kinase C/diolein for 8 min in the presence of ATP and 0.6 \(\mu M\) Ca\(^{2+}\); EGTA was subsequently added to reduce the free Ca\(^{2+}\) concentration to 80 nM, and Ca\(^{2+}\) uptake rates were then measured during the next 20 min. This procedure was designed to mimic the Ca\(^{2+}\) transients observed in a stimulated cell or in one permissibilized to Ca\(^{2+}\). Whereas diolein was ineffective in supporting protein kinase C stimulation at constant exposure to 80 nM free Ca\(^{2+}\), brief exposure to 0.6 \(\mu M\) Ca\(^{2+}\) enabled diolein to activate the transport system when Ca\(^{2+}\) was then reduced to 80 nM (Fig. 8). Preexposure of IOV to elevated Ca\(^{2+}\) did not significantly affect rates of Ca\(^{2+}\) transport observed in the
Protein Kinase C Regulation of the Ca²⁺ Pump

**FIG. 9. Effect of activators on the purified erythrocyte Ca²⁺-ATPase.** Ca²⁺-dependent rates of enzymatic ATP hydrolysis were measured in the presence of 5 mM Mg²⁺, 100 μM ATP, and 100 μM free Ca²⁺. Activity in the presence of Mg²⁺ alone was subtracted. a, enzyme with no added activators (mean ± range of two experiments); b, with 7.5 μg/ml calmodulin (mean ± S.D., three experiments); c, with 120 units/ml protein kinase C (PKC) and 100 nM TPA (mean ± S.D., three experiments); d, with 120 units/ml protein kinase C and 0.5 μg/ml diolein (DG) (mean ± range of two experiments); e, with protein kinase C, 100 nM TPA and 7.5 μg/ml calmodulin (mean ± S.D., three experiments); f, with protein kinase C, 0.5 μg/ml diolein and 7.5 μg/ml calmodulin (single experiment). In c-f, phosphatidylserine was included at 4 μg/ml to maximally activate protein kinase C. Neither PS at this concentration nor TPA alone affected the basal rates of Ca²⁺-dependent ATP hydrolysis.

**DISCUSSION**

In this investigation, the activated form of purified protein kinase C was found to stimulate Ca²⁺ transport in alkaline phosphatase-treated IOV (Figs. 1–8). Significant effects were observed at physiologically relevant concentrations of free Ca²⁺: 0.08–5 μM (Figs. 2–5 and 8). Protein kinase C stimulated Ca²⁺ transport only in the presence of the specific activators phorbol ester or diacylglycerol. These properties reflect the activation characteristics of purified protein kinase C reported previously by others. Treatment of protein kinase C-activated IOV with alkaline phosphatase completely reversed stimulation of Ca²⁺ uptake, consistent with a mechanism involving protein phosphorylation.

Protein kinase C and phorbol ester increased the maximum velocity of erythrocyte IOV Ca²⁺ transport but had no significant effect upon the apparent Kₘ for Ca²⁺ (Figs. 5 and 6). In contrast, calmodulin increased both the maximum velocity and the Ca²⁺ sensitivity of the pump (Fig. 6). A combination of the two activators (protein kinase C/TPA or protein kinase C/diolein, plus CaM) showed additive effects at maximal doses of each (Fig. 5), and therefore calmodulin and protein kinase C may be said to exert their effects through different mechanisms.

Either protein kinase C/TPA or protein kinase C/diolein were found to stimulate the activity of the purified calcium pump ATPase (Fig. 9), just as they stimulated Ca²⁺ transport in intact vesicles. Therefore, it appears that protein kinase C acts directly upon the calcium pump and not indirectly through intermediate effectors. Preliminary results indicate that protein kinase C does, in fact, phosphorylate the purified calcium pump (not shown); this is now being investigated further.

Alkaline phosphatase-treated IOV were found to exhibit far more consistent Ca²⁺ transport rates than nontreated vesicles. In the present experiments on alkaline phosphatase-treated IOV, CaM was definitely found to activate Ca²⁺ transport by CaM has been reported (11,12). In contrast with other reports (41,42), CaM was definitely found to activate Ca²⁺ transport rates never exceeded those obtained with in vitro addition of maximal protein kinase C/TPA to phosphatase-treated IOV. The rates were occasionally equal, in which case no stimulation by protein kinase C/TPA was observed. This suggests that protein kinase C may have been the endogenous activating factor in normal membranes. In fact, human erythrocytes do contain protein kinase C (23) and can produce diacylglycerol by Ca²⁺-stimulated breakdown of phosphatidylinositol (38,39). Erythrocytes have been shown to be permeabilized to Ca²⁺ by sheer stress (40), and thus blood-drawing and handling procedures may induce activation of endogenous protein kinase C.

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indicates that treatment of intact cells with phorbol ester to activate protein kinase C may alter basal Ca^{2+} transport activity.

Diolein was as effective as TPA in supporting protein kinase C stimulation of Ca^{2+} transport at 0.6 μM free Ca^{2+} (Fig. 3). However, diolein was ineffective in supporting protein kinase C stimulation at 80 nM free Ca^{2+}, unless IOV in the presence of protein kinase C/diolein were first exposed to a transient elevation of Ca^{2+} to 0.5 μM (Fig. 8). If the red cell Ca^{2+}-ATPase is truly representative of the plasmalemmal Ca^{2+}-ATPase in other cell types, these results are important for two major reasons. First, as diacylglycerol is the natural agent stimulating protein kinase C in intact cells, the calcium pump is probably not stimulated by protein kinase C in resting cells. Second, the concentration of diacylglycerol in the plasma membrane rises in hormonally stimulated cells during and after a cytosolic Ca^{2+} transient (6, 43). As shown by the experiments reported here, diolein can induce protein kinase C stimulation of erythrocyte plasma membrane Ca^{2+} transport after brief exposure of the membrane to elevated Ca^{2+}, even when the Ca^{2+} concentration then returns to basal levels (Fig. 8). Together, these observations suggest that in an intact cell, protein kinase C activation of Ca^{2+} extrusion occurs only during the posttransient phase of cell stimulation. Because the effects of CaM and protein kinase C are then additive, the activity of the calcium pump would be higher in the post-Ca^{2+} transient phase than in a resting cell.

A new model of the erythrocyte plasma membrane calcium pump is thus presented in Fig. 10. In an intact cell in its basal state, calmodulin may bind to and activate the Ca^{2+} pump to some degree. When intracellular Ca^{2+} is elevated, CaM further stimulates pump activity. Elevation of cytosolic Ca^{2+}, in combination with production of diacylglycerol, enables endogenous protein kinase C to bind to the plasma membrane. This membrane-associated protein kinase C, in turn, phosphorylates the Ca^{2+} pump protein and further activates the pump. Excess cytosolic Ca^{2+} may then be extruded, and the free Ca^{2+} falls toward basal levels both CaM and protein kinase C enable the pump to remain in an activated state. One would predict that upon removal of agonist, when influx of Ca^{2+} ceases and diacylglycerol is no longer produced, the Ca^{2+}-ATPase would be dephosphorylated by an endogenous phosphatase as yet uncharacterized. Efflux of Ca^{2+} through the calcium pump would return to its original slow rate.

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