Protein Kinase C Activation Induces Phosphatidylserine Exposure on Red Blood Cells†

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ABSTRACT: We have shown previously that red blood cells (RBCs) can be induced to influx Ca^{2+} when treated with lipid mediators, such as lysophosphatidic acid and prostaglandin E_2, that are released during clot formation. Since calcium loading of RBCs can lead to both protein kinase C (PKC) activation and phosphatidylserine (PS) exposure, we decided to investigate the possible linkage between PKC activation and membrane PS scrambling using phorbol 12-myristate-13-acetate (PMA), a commonly used activator of PKC. Treatment of RBCs with PMA in a calcium-containing buffer caused immediate PS exposure in an RBC subpopulation. The size of the subpopulation did not change upon further incubation, indicating that not all RBCs are equally susceptible to this treatment. Using a fluorescent indicator, we found a subpopulation of RBCs with elevated intracellular calcium levels. In the absence of extracellular calcium, no PS exposure was found. However, we did find cells with high levels of calcium that did not expose PS, and a variable percentage of PS-exposing cells that did not show elevated calcium concentrations. Inhibition of PKC with either calphostin C, a blocker of the PMA binding site, or chelerythrine chloride, an inhibitor of the active site, diminished the level of formation of PS-exposing cells. However, the inhibitors had different effects on calcium internalization, indicating that a high calcium concentration alone was not responsible for inducing PS exposure in the absence of PKC activity. Moreover, PKC inhibition could prevent PS exposure induced by calcium and ionophore treatment of RBCs. We conclude that PKC is implicated in the mechanism of membrane phospholipid scrambling.

Red blood cells (RBCs)[1] are generally considered to be biologically inert and inactive in intercellular communication. Thus, the presence of RBCs in a thrombus has been commonly attributed to passive entrapment. Some evidence, however, has suggested a more active role of the RBC in hemostasis, indicating that RBCs could be recruited by platelets into the clotting process (*1*, *2*). Activated platelets release several compounds that participate in cross-talk with other cells. These extracellular messengers, such as prostaglandins, adenine nucleotides, platelet-activating factor, and lysophosphatidic acid (LPA), among others, have been shown to activate signal transduction processes in other cells (*3*−*5*). Calcium mobilization is often an important intracellular effect of these processes. In the RBC, there are no major calcium stores, and significant elevation of intracellular Ca^{2+} levels requires increased calcium influx. Previously, we have shown that LPA or prostaglandin E_2 (PGE_2) treatment in vitro increases the Ca^{2+} levels of RBCs (*1*, *2*).

Under normal conditions, membrane phospholipids are distributed asymmetrically across the membrane monolayers, with phosphatidylserine (PS) exclusively located in the inner monolayer (for reviews, see refs *6* and *7*). It is well-established that calcium loading of RBCs leads to scrambling of the membrane phospholipids, resulting in exposure of PS on the surface of the cell (*8*, *9*). This process is possibly mediated by activation of the calcium-dependent phospholipid scramblase (*10*), but PS exposure only occurs when the aminophospholipid translocase, or flippase, is inhibited as well. RBCs that expose PS form a catalytic surface for hemostatic reactions, and thus have prothrombotic characteristics (*7*, *11*).

We hypothesized that the signal transduction processes activated by the factors released by activated platelets could lead to elevated Ca^{2+} levels in RBCs, and thereby activate the phospholipid scrambling process, leading to a prothrombotic RBC that could participate in clot formation. We investigated the impact of the activation of a common signal transduction pathway, mediated through protein kinase C (PKC), on both calcium influx into RBCs and PS exposure. We demonstrate that phorbol 12-myristate-13-acetate (PMA), a commonly used specific activator of PKC, stimulates the influx of calcium into a subpopulation of RBCs, and also causes immediate PS exposure in a subpopulation of RBCs. We conclude that PKC is implicated in the mechanism of membrane phospholipid scrambling, supporting our hypothesis that a subpopulation of RBCs is susceptible to recruit-

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[1] Abbreviations: AV, annexin V; DAG, diacylglycerol; IP_3, inositol triphosphate; LPA, lysophosphatidic acid; NEM, *N*-ethylmaleimide; PGE_2, prostaglandin E_2; PKC, protein kinase C; PMA, phorbol 12-myristate-13-acetate; PS, phosphatidylserine; RBC, red blood cell.

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ment by platelets through activation of signal transduction processes.

MATERIALS AND METHODS

Materials. Phorbol 12-myristate 13-acetate (PMA), chelerythrine chloride, and calphostin C were obtained from Calbiochem (La Jolla, CA). Radiolabeled PMA ([20-^{3}H(N)]-phorbol 12-myristate 13-acetate) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Annexin V conjugates were prepared as described previously from recombinant annexin V (*12*), using several protein labeling kits from Molecular Probes (Eugene, OR).

PMA Treatment of RBCs. Whole blood was obtained from healthy donors after informed consent had been obtained. No particular donor selection was made for any of the experiments. RBCs were isolated by resuspension in buffer H [10 mM HEPES (pH 7.4) and 145 mM NaCl] and centrifugation followed by aspiration of the supernatant. RBCs were resuspended at 0.8% hematocrit in buffer H containing 2 mM $CaCl_2$. Where indicated, PKC inhibitors were added from stock solutions (at least 200-fold greater than the indicated final concentration) in dry DMSO to this suspension, followed by a 30 min pretreatment at room temperature. ATP depletion was carried out by incubation for 2 h at 37 °C in a buffer containing 10 mM HEPES, 130 mM NaCl, 2.7 mM KCl, 1 mM EGTA, 10 mM inosine, 6 mM iodoacetamide, and 5 mM sodium tetrathionate, which reduced the ATP content to <2% of its normal level as described previously (*13*).

PMA was dissolved at 1 mM in ethanol. Aliquots of this solution were dispersed at twice the final concentration in buffer H containing 2 mM $CaCl_2$. From this solution, 200 μL was added to 200 μL of the 0.8% cell suspension, followed by brief mixing and incubation at room temperature without further stirring. Unless noted otherwise, the routine incubation time was 30 min with 6 μM PMA. These conditions for the incorporation of PMA were carefully maintained to improve the reproducibility (see below).

Incorporation of PMA into RBCs. Because of its hydrophobicity, PMA is likely to partition onto any hydrophobic surface, including the incubation container, the suspension, and the cells. To assess the reproducibility of the amount of supplemented PMA that actually was incorporated into the RBC membrane, we used ^{3}H-radiolabeled PMA (American Radiolabeled Chemicals Inc.). RBCs were incubated with 0.71 Ci/mmol of [^{3}H]PMA at 2, 4, 6, and 8 μM PMA (final concentrations) using the routine procedure described above. Following a 20 min PMA incubation, cells were separated from the medium by centrifugation, and analysis of the amount of radiolabel in each fraction was performed. Subsequently, the tubes in which the incubation had taken place were washed out with scintillation fluid to analyze the percentage of PMA lost by adherence to the tube surface.

Ionophore Treatment of RBCs. Washed RBCs were pretreated with 10 mM *N*-ethylmaleimide (NEM) for 30 min at 37 °C to inhibit the flippase. NEM-treated cells were resuspended at 0.8% hematocrit in buffer H containing 2 mM $CaCl_2$. Where indicated, calphostin C was added to this suspension from a stock solution in dry DMSO, followed by a 30 min pretreatment at room temperature. Calcium ionophore A23187 was dissolved at 100 μM in ethanol, and added to the RBCs at a final concentration of 0.4 μM, followed by incubation for 30 min at 37 °C. An aliquot of the treated cells was labeled with annexin V as described below.

RBC Labeling and Flow Cytometry. We first assessed the time-dependent progress of PS exposure during PMA incubation. For this purpose, FITC-conjugated annexin V (FITC−AV) was present at 60 ng/mL during the incubation, and at indicated time intervals, 50 μL aliquots were transferred to 450 μL of buffer H containing 2 mM $CaCl_2$, and analyzed by flow cytometry.

For further routine procedures, annexin V was not present during PMA incubation. At the end of the incubation, 50 μL of the cell suspension was transferred to 450 μL of buffer H containing 2 mM $CaCl_2$ and annexin V. Either 40 ng/mL Alexa-conjugated annexin V (Al−AV) or 70 ng/mL biotin-conjugated annexin V (Bi−AV) in combination with 1 μg/mL R-phycoerythrin-conjugated streptavidin was used, with similar results. The incubation was continued for 10 min followed by flow cytometric analysis. Data acquisition was performed on a Becton Dickinson FACS Calibur, and analysis was carried out with CellQuest software (Becton Dickinson, San Jose, CA) as described previously (*9*). Cells that exhibited an increased fluorescence upon incubation with fluorescently labeled annexin V were identified as cells that expose PS (*9*).

Detection of Intracellular Ca^{2+}. The presence of cells with increased intracellular levels of Ca^{2+} was assessed using the commonly used fluorescent probe Fluo3-AM and flow cytometry. Fluo4-AM was used in some of the experiments as a substitute for Fluo3-AM, giving brighter fluorescence. All data that indicate the use of Fluo3 were redetermined in final experiments with Fluo4 with identical quantitative results. RBCs (0.4% hematocrit) were loaded with 1 μM Fluo4-AM for 1 h at 37 °C as described previously for Fluo3-AM (*9*), prior to inhibitor and PMA treatment as described above. Fluo3 was used in combination with Bi−AV labeling to test the correlation between increased levels of intracellular Ca^{2+} and PS exposure in PMA-treated cells. To establish the Fluo3 fluorescence intensity of RBCs with high intracellular Ca^{2+} levels, RBCs were loaded with Ca^{2+} by treatment for 5 min at room temperature with the calcium ionophore A23187 (0.2 μM) at 0.04% hematocrit in the presence of 2 mM $CaCl_2$.

RESULTS

RBCs treated with PMA at a concentration of >1 μM showed rapid formation of an apparent subpopulation of PS-exposing cells in the presence of extracellular calcium (Figure 1). In the absence of calcium, no PS exposure was induced (data not shown). PS exposure did not appear when calcium was added after a 20 min pretreatment with PMA, indicating that the sensitivity to PMA stimulation was transient and required elevated intracellular Ca^{2+} levels.

The size of the PS-exposing subset was dependent on the PMA concentration, and reached an apparent plateau after incubation for 10 min, indicating that only a subpopulation of RBCs is susceptible to this effect at each PMA concentration. Repeated exposure to PMA did not increase the size of the subset. These data also indicate that PS exposure is virtually immediate after addition of PMA, taking into

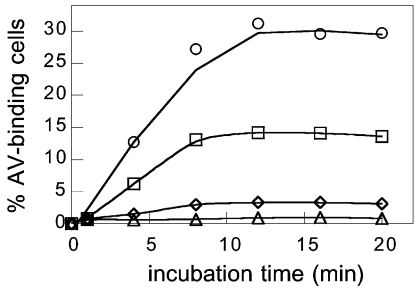

FIGURE 1: PMA induces PS exposure in a subpopulation of RBCs. RBCs at 0.4% hematocrit were treated with PMA at concentrations of 6 (○), 3 (□), 1.5 (◇), and 0 μM (△) at room temperature in the presence of FITC−AV. At the indicated times, aliquots were analyzed by flow cytometry. The experiments were performed on the same day with blood from the same donor. Similar experiments showed the same pattern, but with variations in the size of the subpopulation responding to the treatment.

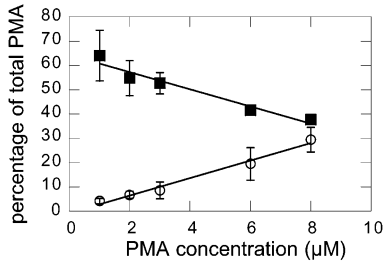

FIGURE 2: Incorporation of [^{3}H]PMA into the RBC membrane. RBCs were labeled with [^{3}H]PMA using the same procedure employed in all other experiments. The cell fraction (■) and supernatant fraction (○) were separated by centrifugation, and the radiolabel in each fraction was quantified. The data that are shown, representing the percentage of total PMA found in each fraction, constitute the average ± SD for three similar experiments with blood from different donors.

account the fact that annexin binding takes ~10 min to complete (data not shown). Repeat experiments with the same RBCs gave virtually identical results; however, multiple similar experiments with 6 μM PMA using RBCs from different blood donations yielded large variations in the size of the PS-exposing subpopulation [mean ± standard deviation (SD), 13.0 ± 11.6% (n = 60)].

To determine whether the variability was due to variations in incorporation of the probe, we used radiolabeled PMA and assessed the relative uptake of this probe by the cells. The data in Figure 2 show that at a total PMA concentration of 6 μM only 41.5 ± 1.2% (n = 3) of the PMA was located in the cell. Of the total PMA, 70 ± 5% was found in supernatant and pellet together, indicating that some PMA was bound to the glass tube or to the plastic devices used in the procedure. On the other hand, the variation in these data was very small, showing that the procedure was very consistent from experiment to experiment when incubation conditions were kept the same. Thus, it seems that the variation observed in the size of the PS-exposing subset indicates susceptibility differences between different blood samples, rather than a variation in incorporation of the PMA.

To verify that the effect of PMA on PS exposure was indeed mediated by PKC, we used several inhibitors. Calphostin C, an irreversible inhibitor of the DAG-binding site of PKC, completely abolished the PS exposure induced by PMA, as shown in Figure 3. Chelerythrine chloride, an inhibitor of the active (phosphorylation) site of PKC,

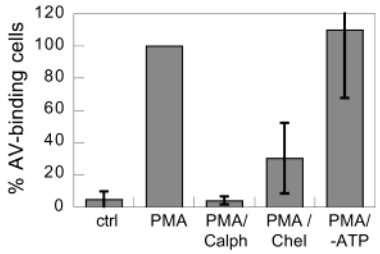

FIGURE 3: PKC inhibitors abolish the effect of PMA on PS exposure. ATP-depleted cells (−ATP) or cells pretreated with 1 μM calphostin C (Calph) or in the presence of 10 μM chelerythrine chloride (Chel) were treated with 6 μM PMA for 30 min, followed by incubation with AV for 10 min and FACS analysis, and compared to untreated cells (ctrl) and cells treated with PMA only (PMA). For each experiment, the data that were found were expressed as a percentage of the data found with PMA treatment only (set at 100%) obtained with the same blood sample. Indicated are averages ± SD of at least three similar experiments with blood from different donors.

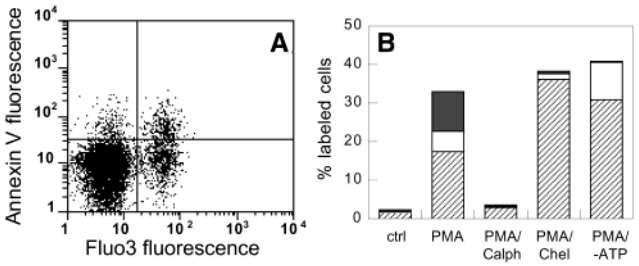

FIGURE 4: High intracellular calcium levels in a subpopulation of PMA-treated RBCs. RBCs were loaded with Fluo3 or Fluo4 and treated with inhibitor, followed by standard PMA treatment and labeling with biotin−AV and PE−streptavidin. (A) Typical pattern of fluorescence distribution in arbitrary units (AU) in the two channels of the flow cytometer after PMA treatment, shown for one specimen not treated with any inhibitor. Data were similar to those of all other experiments (n = 50). (B) Percentage of events in each quadrant of the plot in panel A as obtained in the absence of PMA and inhibitors (ctrl), with calphostin C (calph), with chelerythrine chloride (chel), or after ATP depletion (−ATP). The lower right quadrant (hatched portion of the bar) represents the percentage of cells only labeled with Fluo3 (or Fluo4), the upper right quadrant (empty portion of the bar) cells labeled with Fluo3 (or Fluo4) and Bi−AV, and the upper left quadrant (filled portion of the bar) those cells only labeled with Bi−AV. Data represent the average for at least three identical experiments.

inhibited 70% of the PS exposure. This indicates that the PMA-induced effect was not due simply to pore formation or perturbation of the membrane, but that binding to PKC was essential. To investigate whether the PMA-induced PS exposure was dependent on intracellular ATP, we depleted RBCs of ATP by a method that routinely produces ATP levels of less than 2% of the original levels (*9*). As also shown in Figure 3, this treatment did not prevent PMA-induced PS exposure, indicating that little or no ATP is required for the scrambling process.

We used Fluo3, a probe commonly used to detect increased levels of intracellular calcium, in combination with Bi−AV and PE−SA to see if the calcium concentrations were elevated in the PS-exposing cells. As shown in Figure 4A, a subset indeed had elevated Fluo3 fluorescence (right upper and lower quadrants). However, of the PS-exposing cells (increased annexin V fluorescence, right and left upper quadrants), on average only 68% showed increased calcium levels after a 20 min incubation with PMA (right upper

quadrant). The low-calcium PS-exposing population (left upper quadrant) was smaller when the incubation time was shorter (not shown). ATP depletion did not reduce the PMA-induced increase in calcium level or PS exposure. However, in this case, virtually all PS-exposing cells also experienced an increase in the level of intracellular calcium, and no low-calcium PS-exposing population was present. These data suggest that calcium was pumped out of these cells by the Ca-ATPase, but only after a previous transient increase in the level of calcium had led to PS exposure.

The distribution of cells between the different flow cytometric quadrants as shown in Figure 4A is further depicted in Figure 4B. This figure shows the percentage of cells found in three of the four quadrants. The hatched portion of the bars indicates cells that are fluorescent in the Fluo3 channel as the result of increased calcium levels (lower right quadrant), but do not expose PS indicated by low annexin V fluorescence. The empty portion of the bar represents the percentage of cells that expose both PS and have high calcium levels (upper right quadrant). The filled portion of the bars shows the percentage of cells that do expose PS but do not have elevated calcium levels (upper left quadrant). PMA incubation leads to a large population of calcium-loaded cells that does not expose PS, indicating that PMA-induced calcium influx does not induce PS exposure in every RBC (hatched portion of the bars). As shown in Figure 4B, PKC inhibition with calphostin C prevented most of the calcium internalization (sum of the empty and hatched bars). However, chelerythrine chloride did not prevent this PMA-induced increase in the level of calcium, and even led to a higher percentage of cells with increased levels of calcium than with PMA alone. This inhibitor, however, decreased the percentage of PMA-induced PS-exposing cells. Both inhibitors had no effect on calcium influx and PS exposure when the cells were not treated with PMA (data not shown). These data indicate that calcium internalization does not necessarily lead to PS exposure.

This led us to hypothesize that loading cells with calcium in the presence of PKC inhibitors would prevent the normally induced PS exposure. To investigate this, RBCs were first pretreated with NEM to inhibit the flippase, and further incubated in the presence of calcium ionophore A23187 and 2 mM $CaCl_2$. This resulted in a PS-exposing subpopulation of $67 \pm 13\%$. However, when the cells were pretreated with calphostin C, this subpopulation was reduced to less than 1%. These data indicate that the increase in the level of cytosolic calcium by itself is not sufficient for the loss of PS asymmetry, even in cells that are permeable for calcium. PKC stimulation seems to be essential for PS exposure, and our data indicate that PKC stimulation induces membrane phospholipid scrambling, and PS exposure in a subpopulation of RBCs.

DISCUSSION

We have shown that the activation of PKC with PMA, a compound commonly used for this purpose, leads to PS exposure in a subpopulation of RBCs. The PS-exposing subpopulation is diminished in size during PMA treatment in the presence of two different inhibitors, calphostin C, which inhibits the PMA-binding regulatory site of PKC, and chelerythrine chloride, which inhibits the catalytic (phosphorylating) site of PKC. These data indicate that the PS-exposing effect of PMA treatment is specific and indeed PKC-mediated.

The presence of PKC in RBCs has been reported previously (*14*), and several of its targets for phosphorylation have been described (*15*). In most cell types, PKC is stimulated after receptor-induced generation of the second messengers inositol 3-phosphate (IP_3) and diacylglycerol (DAG), a cofactor for PKC. IP_3 releases Ca^{2+} from intracellular stores, mainly, the endoplasmatic reticulum, and this process is thought to translocate PKC to the membrane where DAG binding takes place for full activation of the enzyme. Although a similar IP_3-induced calcium release is unlikely to function as the second messenger in RBCs since no endoplasmic reticulum is present, it has been reported that enhanced intracellular calcium levels can induce PKC activation in RBCs (*16*). Moreover, PKC has been shown to translocate to the membrane of RBCs during PMA incubation (*14*, *15*, *17*).

PS exposure generates a catalytic surface for activation of both the tenase complex (factors VIIIa and IXa) and the prothrombinase complex (factors Va and Xa), leading to enhanced generation of thrombin and clotting (*11*). Thus, RBCs that expose PS could assist platelets in clot formation. While our results suggest that activation of signal transduction pathways in the RBC could lead to prothrombotic characteristics of these cells, the signals and mechanisms that play a role in this process remain unclear and are the scope of our ongoing studies.

It is remarkable that PS is not exposed in all cells, but only in an apparent subpopulation that varies significantly in size. Since hydrophobic compounds such as PMA distribute themselves over all hydrophobic surfaces, including incubation containers, we used identical incubation conditions for all experiments to reduce the variation in the extent of PMA incorporation induced by the procedure. Our data indicate that the variations in the amount of PMA taken up by RBCs were minimal between experiments. Moreover, repeat experiments using the same RBCs resulted in similar PS-exposing subpopulations. However, the use of RBCs from different donations resulted in significant variability. This suggests that the variability that we find in response to PMA is an intrinsic property of different RBC specimens. Apparently, not all RBCs are equally susceptible to PKC-mediated scrambling. It is possible that the expression level and remaining activity of PKC isoenzymes in RBCs vary among subsets of these cells. It has been established that prolonged stimulation of cells with PMA, in fact, results in cleavage and deactivation of PKC, possibly by calpain (*17*, *18*). The various PKC subtypes show a different sensitivity to this deactivation (*19*), but reportedly, RBCs seem to contain only PKC α, ι, μ, and ζ (*20*−*22*). PKC α is the only phorbol ester-responding subtype reported to be present in RBCs and thus applicable to this study. PS exposure as a result of PMA treatment is likely due to activation of PKC and not deactivation, since the timing of the appearance of PS is rapid after PMA addition, and no PS exposure is observed when calcium is added after the initial stimulation. This, and the fact that a plateau is reached by 10 min after PMA addition, may indicate deactivation of PKC after this incubation time, possibly resulting in the arrest of the formation of PS-exposing cells and in a concentration-dependent limitation

of the number of cells affected. Moreover, reduced PKC activity has been found in the densest, and hence oldest, fraction of rabbit erythrocytes (*23*), supporting the idea that not every RBC has the same PKC content and that differences in response to PMA-induced activation can be expected. Further studies are required to determine the activation and deactivation of the PKC subtypes involved in relation to the occurrence of PS exposure.

Exposure of PS becomes apparent only when both the aminophospholipid translocase (flippase) is impaired and the phospholipids are scrambled by an additional mechanism (*7*). The recently cloned calcium-dependent scramblase (*10*) is likely to play a role in this mechanism. It is unclear how PKC activation modulates both of these processes, but our data do not support the simple explanation that increased intracellular calcium levels following PKC activation lead to direct activation of the scramblase and inhibition of the flippase. We used millimolar levels of calcium in our studies to mimic external conditions present in plasma. Extracellular calcium was required for PMA-induced PS exposure, and our studies show that indeed PMA stimulation leads to calcium influx in a subpopulation of RBCs. The actual intracellular concentrations achieved under these conditions cannot be determined by using the fluorescent calcium indicators, Fluo3 or Fluo4, as these compounds gain their maximum fluorescence at sub-micromolar intracellular calcium levels. However, the intracellular calcium concentrations induced by PMA are likely not in the millimolar ranges as this seems to be incompatible with normal physiology. On the other hand, such millimolar levels are achieved by using the calcium ionophore, which leads to a complete equilibrium of calcium across the membrane. The mechanism for the PMA-induced calcium influx is unclear, but points to a temporary imbalance between calcium influx and the active outward directed calcium pump, which normally keeps intracellular calcium in the RBC at nanomolar levels. Enhanced intracellular calcium levels can induce PKC activation in the RBC, implying that the initially enhanced calcium levels could recruit more PKC to translocate to the membrane and exert positive feedback. Reduction of the ATP content to less than 2% (less than 30 μM) clearly had a positive effect on the percentage of PS-exposing cells found with a higher calcium content, indicating that the ATP-dependent calcium pump was inhibited under those conditions and calcium levels were not reduced as found in some ATP-replete PS-exposing cells. These reduced ATP levels, however, did not appear to influence the involvement of PKC in calcium influx and scrambling, although diminished PKC activity would be expected on the basis of its reported K_m for ATP of 13-62 μM (*24*, *25*). This finding argues in favor of the possibility that PMA-induced calcium influx is independent of PKC phosphorylation. This is further supported by the fact that chelerythrine chloride, which acts on the active site of PKC, does not lead to a reduction in calcium influx. In contrast, calphostin C, which interferes with the PMA binding site of PKC, reduces calcium influx. Together, these data suggest that calcium influx is dependent on the binding of PMA to PKC under conditions that do not favor phosphorylation of a substrate. The specifics of this process are to be determined.

Despite the fact that extracellular calcium was required for PS exposure in our experiments, and that the intracellular calcium levels increased in a subpopulation of RBCs during PMA incubation, we did not find a clear correlation between elevated calcium levels and PS exposure. First, a large subset of the PS-exposing cells did not exhibit elevated calcium levels. This population could be derived from cells that extruded this Ca^{2+} after PS had already been exposed. Indeed, it has been reported that the RBC calcium pump is more active after PKC activation (*26*). This explanation is supported by the fact that the low-calcium PS-exposing population was not induced in the absence of ATP, and was smaller when the incubation time was shorter. Second, the percentage of cells containing a high calcium concentration did not decrease in the presence of chelerythrine, although PS-exposing cells were not formed in the presence of this inhibitor. Third, PS exposure was not found if calcium was added after an initial PMA incubation that presumably causes complete deactivation of PKC (*17*). Hence, addition of calcium, after PKC stimulation has terminated, results in the prevention of PS exposure, indicating that PKC stimulation is directly important for PS exposure. Finally, calphostin C was able to inhibit PS exposure induced by loading RBCs with calcium after inhibition of the flippase, which normally results in PS exposure in virtually all RBCs, arguing that calcium by itself is not sufficient to activate the scramblase and inhibit the flippase.

Taken together, these data are in line with the concept that PKC activation leads to increased calcium influx into the cell, as well as activation of phospholipid scrambling by a calcium-independent mechanism, which leads to PS exposure on the cell surface. This finding supports the hypothesis that signaling molecules, possibly released by activated platelets, could contribute to the formation of procoagulant RBC surfaces, which thus could assist platelets in the clotting process.

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