Interplay between Calcium, Diacylglycerol, and Phosphorylation in the Spatial and Temporal Regulation of PKCa-GFP*

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The function of protein kinase C (PKC) is closely regulated by its subcellular localization. We expressed PKCα fused to green fluorescent protein (PKCα-GFP) and examined its translocation in living and permeabilized cells of the human parotid cell line, HSY-EB. ATP induced an oscillatory translocation of PKCα-GFP to and from the plasma membrane that paralleled the appearance of repetitive Ca2+ spikes. Staurosporine attenuated the relocation of PKCα-GFP to the cytosol and caused a stepwise accumulation of PKCα-GFP at the plasma membrane during ATP stimulation. Diacylglycerol enhanced the amplitude and duration of the ATP-induced oscillatory translocation of PKCα-GFP. Ionomycin induced a transient translocation of PKCα-GFP to the plasma membrane despite the continuous elevation of cytosolic Ca2+. The ionomycin-induced transient translocation of PKCα-GFP was prolonged by staurosporine, diacylglycerol, and phorbol myristate acetate. Experiments using permeabilized cells showed that staurosporine or the elimination of ATP and Mg2+ decreases the rate of dissociation of PKCα-GFP from the membrane. Diacylglycerol slowed the dissociation of PKCα-GFP from the membrane regardless of the Ca2+ concentration. The effect of diacylglycerol was attenuated by ATP plus Mg2+ at low concentrations of Ca2+ (<500 nm) but not at high concentrations of Ca2+ (>1000 nm). These data suggest a complex interplay between Ca2+, diacylglycerol, and phosphorylation in the regulation of the membrane binding of PKCα.

Protein kinase C (PKC) isoforms make up a large family of serine/threonine protein kinases and play a key role in transducing numerous signals generated by growth factors, hormones, and neurotransmitters (1–3). All of the isoforms identified to date share in common an amino-terminal regulatory domain. We expressed GFP-tagged PKCα (PKCα-GFP) in HSY-EB cells and examined its translocation in living and permeabilized cells to more directly and quantitatively analyze the interplay of Ca2+, DAG, and phosphorylation in the membrane binding of PKCα.

EXPERIMENTAL PROCEDURES

Reagents—1,2-Dihecanoyl-sn-glycerol (DiC6) was obtained from Sigma. 1,2-Dioctanoyl-sn-glycerol (DiC8) was from Bionol (Plymouth Meeting, PA). Staurosporine and phorbol 12-myristate 13-acetate (PMA) were from Wako Pure Chemicals (Osaka, Japan). Ionomycin was from Calbiochem-Novabiochem. These reagents were dissolved in dimethyl sulfoxide as 200 mM stock solutions and diluted to the desired final concentrations shortly before the experiment. [γ-32P]ATP was from Institute of Isotopes Co. (Budapest, Hungary).

Media—Hanks’ balanced salt solution with Hepes (HBSS-H) contained 137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.41 mM MgSO4, 0.49 mM MgCl2, 0.34 mM NaH2PO4, 0.44 mM NaHPO4, 5.5 mM glucose, 20 mM Hepes-NaOH, pH 7.4, and 1% bovine serum albumin (BSA). Intracellular-like medium (ICM) contained 125 mM KCl, 19 mM NaCl, 10 mM Hepes-KOH, pH 7.5, 100 μg/ml saponin, EGTA, and appropriate concentrations of CaCl2. ICM-250S and ICM-1000S solutions consisted of ICM containing 250 and 1000 nM free Ca2+.

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tosyl-t-phenylalanine chloromethyl ketone, 30 μM peptatin A, 30 μM leupeptin, 300 μg/ml soybean trypsin inhibitor, and 3% Triton X-100. The 1× stop solution was a 1:2 mixture of 3× stop solution and ICM-250S.

Cell Culture and Transfection—The HSY human parotid cell line, a generous gift from Dr. Mitsubou Sato (Tokushima University, Japan), was subcloned by using a dilution plating technique, and six clones were obtained. One of these subclones, HSY-EB, was used for imaging experiments in which the cells were cultured for 1 day in sample chambers consisting of 7 × 7-mm plastic cylinders glued to round glass coverslips with silicone rubber adhesive. Transient transfections were performed using a CaPHos mammalian transfection kit (CLONTECH, Palo Alto, CA) with 0.4 μg/ml plasmid (pPKCα-EGFP or pEGFP-C3; CLONTECH) according to the manufacturer’s instructions.

Stable transfections of HSY-EB cells with the pPKCα-EGFP vector were established using LipofectAMINE 2000 (Invitrogen). G418 selection (0.5 mg/ml) was initiated 24 h after the transfection. After 1 week, cells were expanded for an additional week without G418. Colonies of PKCα-GFP-expressing cells were screened by fluorescence microscopy. One of the stable HSY-EB transfectants (PKCα-GFP/EB2 cells) was used in phosphorylation experiments. All cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium supplemented with 10% newborn calf serum, 2 mM glutamine, and 100 μg/ml each of penicillin and streptomycin (all from Invitrogen) as described previously.

Visualization of PKCα Translocation and [Ca2+]i in Intact Cells—Experiments were carried out on cells cultured for at least 3 days after transfection. Cells were incubated with 3 μM Fura Red/AM (Molecular Probes, Eugene, OR) in HBSS-H for 30 min at room temperature. After loading Fura Red, the cells were washed and incubated in HBSS-H without BSA. Drug addition was performed by replacing HBSS-H buffer with experimental buffers containing various reagents.

The distribution of PKCα-GFP and [Ca2+]i were monitored simultaneously with confocal microscopy using a Leica TCS SP system (Leica, Heidelberg, Germany) equipped with a 40× PL Fluotar objective. Confocal images were obtained by excitation at 488 nm and dual emission wavelengths at 495–550 nm for Fura and at 600–700 nm for Fura Red. Time series of 50–150 confocal images were recorded for each experiment at time intervals of between 2.5 and 10 s. Unless stated otherwise, experiments were performed independently on at least four different occasions, and in each experiment, recordings were obtained from 2–10 cells.

Analysis of the Dissociation of PKCα-GFP in Permeabilized HSY-EB Cells—The dissociation of PKCα-GFP was assessed using saponin-permeabilized HSY-EB cells in ICM. The concentration of free Ca2+ in the medium ([Ca2+]i) was calculated from the pH and the amount of potassium, sodium, calcium, EGTA, ATP, and magnesium by the method of Fabiato and Fabiato (18). In this experiment, PKCα-GFP-transfected cells first were permeabilized with ICM-1000S. Cells were then exposed to test media consisting of ICM with variable [Ca2+]i, prewarmed at 4°C. The HSY-EB cells were expanded for an additional week without G418. Colonies of PKCα-GFP-expressing cells were screened by fluorescence microscopy. One of the stable HSY-EB transfectants (PKCα-GFP/EB2 cells) was used in phosphorylation experiments. All cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium supplemented with 10% newborn calf serum, 2 mM glutamine, and 100 μg/ml each of penicillin and streptomycin (all from Invitrogen) as described previously.

RESULTS

ATP-induced Oscillatory Translocation of PKCα-GFP—The stimulation of phospholipase C-coupled receptors results in the generation of inositol 1,4,5-trisphosphate-induced Ca2+ signals and DAG signals. Our previous study demonstrated that the activation of P2y-purinoceptors induces a typical Ca2+ oscillation in HSY cells (19). Ca2+ and DAG are known as regulators for the translocation of cPKCs. We therefore examined the dynamic translocation of PKCα-GFP during ATP-induced Ca2+ oscillations. Earlier studies have shown that a C-terminal GFP tag does not affect the catalytic activity or the cofactor dependence of PKCα (15, 20). To monitor the [Ca2+]i and the translocation of PKCα-GFP simultaneously, a calcium indicator, Fura Red, was loaded into PKCα-GFP-expressing HSY-EB cells.

Fig. 1 shows typical images and time courses of the translocation of PKCα-GFP versus the calcium spikes. In unstimulated cells, PKCα-GFP was evenly distributed throughout the cytosol (Fig. 1, a1), but stimulation with 10–100 μM ATP resulted in oscillations in [Ca2+]i (Fig. 1, b1–b4) and c1 and simultaneously oscillatory translocations of PKCα-GFP to and from the plasma membrane (Fig. 1, a1–a4 and c). The extent of the ATP-induced translocation of PKCα-GFP was 15–30% of the maximal translocation induced by ionomycin (Fig. 1, a5). Oscillatory translocation of PKCα-GFP was also observed when the cells were stimulated with 10–100 μM carbacyclol (data not shown). When HSY-EB cells expressing a control plasmid,
EGFP, were loaded with Fura Red, the GFP fluorescence patterns were not changed by ATP, carbachol, or ionomycin (data not shown). Similar oscillatory translocation of fluorescent PKCa fusion proteins has been reported in human embryonic kidney cells stimulated with histamine (16).

**Effect of Staurosporine and DAG on ATP-induced Oscillatory Translocation of PKCa-GFP**—The subcellular localization of cPKCs is known to be affected by DAG (1). In addition, it has been suggested that the kinase activity of PKC itself plays an essential role in the relocation of PKCβII (12, 21). We therefore examined the effect of a membrane-permeable DAG, DiC8, and the PKC inhibitor staurosporine on the ATP-induced oscillatory translocation of PKCa-GFP. Staurosporine itself had no effect on the distribution of PKCa-GFP (Fig. 2b), but when cells were pretreated with staurosporine, PKCa-GFP accumulated at the plasma membrane in a stepwise manner in response to the repetitive ATP-induced Ca2+ spikes (Fig. 2). This result suggests that the oscillatory translocation of PKCa-GFP is driven by the interplay of Ca2+ signals and kinase activities.

The addition of DiC8 to the cells decreased the frequency of the ATP-induced repetitive Ca2+ spiking. This effect of DiC8 is probably due to suppression of phosphoinositide metabolism through the activation of PKCs (19). DiC8 markedly increased the extent of the translocation of PKCa-GFP (Fig. 3, a–g). The traces in Fig. 3, i and j, clearly demonstrate that externally added DiC8 enhanced the amplitude of the oscillatory translocation of PKCa-GFP (Fig. 3i) without any enhancement in the amplitude of the Ca2+ spikes (Fig. 3j). DiC8 alone did not change the localization of PKCa-GFP (Fig. 3c). To the best of our knowledge, this is the first demonstration that the lipid mediator DAG can act as an amplitude modulator for the oscillatory translocation of PKCa. The expanded time course revealed that in the absence of DiC8, PKCa-GFP rapidly dissociated from the plasma membrane when each Ca2+ spike was
terminating (Fig. 3k). In contrast, after the addition of DiC8, the dissociation of PKCα-GFP was delayed after the Ca\(^{2+}\) spike (Fig. 3l). In the presence of DiC8, the half-maximal relocation (recovery of PKCα-GFP fluorescence in the cytosol) was delayed by 5–10 s after the half-maximal drop in [Ca\(^{2+}\)]\(_i\) (recovery of Fura Red fluorescence).

The effects of DiC8 on the ATP-induced Ca\(^{2+}\) oscillation and the oscillatory translocation of PKCα-GFP were analyzed quantitatively (Fig. 4). DiC8 increased the intervals between Ca\(^{2+}\) spikes (Fig. 4a), but the amplitude (Fig. 4b) and duration (Fig. 4c) of each Ca\(^{2+}\) spike was not altered even with the highest concentration (100 μM) of DiC8. These results clearly demonstrate that DiC8 did not alter the pattern of each Ca\(^{2+}\) spike. On the other hand, the amplitude and duration of the PKCα-GFP translocation were increased by DiC8 in a concentration-dependent manner. The delay of the half-maximal relocation of PKCα-GFP after the half-maximal recovery of Fura Red fluorescence was increased by DiC8 at concentrations above 3 μM (Fig. 4d). Similar effects were also observed with another DAG analog, DiC6 (data not shown).

Effects of Staurosporine, DiC8, and PMA on Ionomycin-induced Translocation of PKCα-GFP—To clarify the effect of Ca\(^{2+}\) on the translocation of PKCα-GFP, we manipulated the Ca\(^{2+}\) response using ionomycin-treated cells. The treatment of ionomycin continuously elevated [Ca\(^{2+}\)]\(_i\), and if extracellular Ca\(^{2+}\) was removed, the ionomycin treatment lowered [Ca\(^{2+}\)], to below the resting level (Fig. 5a, closed circles). Unlike the [Ca\(^{2+}\)]\(_i\), response, ionomycin induced a transient translocation of PKCα-GFP, the maximal translocation occurred within 30 s (Fig. 5a2), and 50–90% of the translocated PKCα-GFP relocated to the cytosol within 2 min after the stimulation (Fig. 5a3). This implies the presence of a specific mechanism by which PKCα-GFP relocates to the cytosol during [Ca\(^{2+}\)]\(_i\), elevation. Subsequent removal of extracellular Ca\(^{2+}\) resulted in the complete recovery of PKCα-GFP to the cytosol (Fig. 5a4).

We next examined the effect of staurosporine and DAG on ionomycin-induced translocation of PKCα-GFP. When cells were pretreated with staurosporine, PKCα-GFP translocated to the plasma membrane in response to the ionomycin-induced [Ca\(^{2+}\)]\(_i\), elevation but did not relocate to the cytosol until the removal of extracellular Ca\(^{2+}\) (Fig. 5b). These results confirm that the relocation of PKCα is dependent on PKC kinase activity.

Treatment with DiC8 alone did not change the localization of PKCα-GFP (Fig. 5c). However, in DiC8-pretreated cells that were then stimulated with ionomycin, PKCα-GFP translocated to the plasma membrane and remained there during the period of [Ca\(^{2+}\)]\(_i\), elevation (Fig. 5d), relocating to the cytosol only when Ca\(^{2+}\) had been removed. These results indicate that Ca\(^{2+}\) is required for DiC8 to exert its influence on the translocation of PKCα-GFP. Unlike DiC8, PMA alone induced a slow translocation of PKCα-GFP to the plasma membrane; the PMA-induced translocation became detectable at 5 min or later and was completed at 10–15 min (Fig. 5e). When ionomycin was added 30 s after application of PMA, PKCα-GFP was translocated to the plasma membrane (Fig. 5f) and did not relocate even after the removal of extracellular Ca\(^{2+}\). The fact that the PMA-mediated stable binding of PKCα-GFP to the plasma membrane was established within 2 min in ionomycin-treated cells (compared with 10 min in the absence of ionomycin) suggests that the binding of PMA to PKCα-GFP was markedly accelerated by Ca\(^{2+}\). Recent studies on PKCγ have proposed that cPKC isoforms are sequentially activated by Ca\(^{2+}\) and DAG (6). Based on this well established "sequential model," our results can be explained as follows. The initial binding of Ca\(^{2+}\) to the C2 domain of PKCα-GFP recruits the enzyme to the plasma membrane and makes the C1 domain accessible to DAG. The subsequent binding of DAG to the C1 domain enables the continuous binding of PKCα-GFP to the plasma membrane. Although PMA is a functional analog of DAG, its action is persistent because of its slow dissociation from cell membranes and its metabolic stability (22, 23).

Analysis of the Distribution of PKCα Using Saponin-permeabilized Cells—The data presented above suggest that kinase activities and/or DAG modulate the Ca\(^{2+}\)-dependent translocation of cPKCs. The interaction of these regulatory mechanisms, however, is largely unresolved. To explore the question of how kinase activities and DAG modulate the Ca\(^{2+}\)-dependent binding of PKCα-GFP to biological membranes, we initially attempted to directly examine the effect of Ca\(^{2+}\) on the binding of PKCα-GFP to the plasma membrane using saponin-permeabilized cells. As the Ca\(^{2+}\) concentration within permeabilized cells quickly equilibrates to the Ca\(^{2+}\) concentration of the ICM ([Ca\(^{2+}\)]\(_m\)), we expected that PKCα-GFP would translocate to the plasma membrane upon the permeabilization. In fact, 50–70% of the PKCα-GFP fluorescence translocated to the membrane upon permeabilization in ICM containing 1000 nM Ca\(^{2+}\) and 100 μg/ml saponin (Fig. 6, a, b, and f). The translocated PKCα-GFP stably bound to the membrane for at least 10 min (Fig. 6, c and f) but was rapidly released and lost into medium with the subsequent exposure to "Ca\(^{2+}\)-free" ICM (Fig. 6, d–f) in which [Ca\(^{2+}\)]\(_m\) was calculated to be ~1.5 nM. This Ca\(^{2+}\)-dependent fraction of the change in fluorescence (depicted as dF) directly demonstrates the Ca\(^{2+}\)-dependence of PKCα-GFP in its binding to cell membranes.

We then tested the effect of PMA (Fig. 7a) and staurosporine (Fig. 7b) on the release of PKCα-GFP from the membrane.

![Figure 4](image_url) Effect of DiC8 on ATP-induced Ca\(^{2+}\) oscillation and the corresponding translocation of PKCα-GFP. PKCα-GFP-expressing HSY-EB cells were loaded with Fura Red and stimulated for 60 s with 30 or 100 μM ATP (as control). Cells were then exposed to various concentrations of DiC8 with ATP for 60 or 120 s. Fluorescence of PKCα-GFP (open column) and Fura Red (closed column) were monitored in the cytosolic region of each cell, and the interval (a), amplitude (b), and duration at half-amplitude (c) of each oscillatory change in fluorescence during the first 60 s (CONTROL) and the following 60–120 s were compared. d, the delay in the half-recovery of PKCα-GFP relative to that of Fura Red fluorescence. Results are represented as mean ± S.E. of 20–65 cells.
When permeabilized cells were pretreated for 30 s with 1 μM PMA, the dissociation of PKCα-GFP in the Ca\(^{2+}\)-free ICM was strongly blocked. Similar results were also obtained when cells were permeabilized with streptolysin O (data not shown). This result is expected, given the experiment using intact ionomycin-treated cells (Fig. 5e) where PMA blocked the relocation of PKCα-GFP in the absence of extracellular Ca\(^{2+}\), and strongly suggests that the PMA-dependent binding between the C1 domain of PKCα-GFP and the plasma membrane is preserved in permeabilized cells. Because the effect of PMA on the binding is mediated by the C1 domain of PKCα-GFP, we expected that the effect of DiC8 on PKCα-GFP could also be examined in this permeabilized cell system.

PKCα-GFP was not released from the permeabilized cells in ICM containing 250 nM \([Ca^{2+}]_{\text{c}}\) lacking ATP and Mg\(^{2+}\) but was readily released by the addition of ATP and Mg\(^{2+}\) (Fig. 7b). The addition of either ATP or Mg\(^{2+}\) did not release PKCα-GFP at 250 nM \([Ca^{2+}]_{\text{c}}\) (data not shown). The ATP/Mg-dependent release of PKCα-GFP was strongly blocked by staurosporine (Fig. 7b). These data directly indicate that the kinase activities accelerate the release of PKCα-GFP.

It has been suggested that Ca\(^{2+}\)-mediated membrane binding of cPKCs is attenuated by autophosphorylation (12, 21, 24). Thus, we examined whether the ATP/Mg-dependent release of PKCα-GFP is due to its phosphorylation. To directly determine the phosphorylation of PKCα-GFP, released and membrane-bound PKCα-GFP were immunoprecipitated with anti-PKCα antibody (Fig. 8A), and their incorporation of the \(^{32}\)P from radioactive ATP was analyzed by autoradiography (Fig. 8B). Although the phosphorylation of membrane-bound PKCα-GFP was detected, the additional phosphorylation of the released PKCα-GFP was not recognized. This result suggests that the ATP/Mg\(^{2+}\)-dependent release of PKCα-GFP is not directly mediated by the phosphorylation of PKCα-GFP.

**Fig. 5.** Effect of staurosporine, DiC₈, and PMA on ionomycin-induced translocation of PKCα-GFP. PKCα-GFP-expressing HSY-EB cells were loaded with Fura Red. Control cells (a) and cells pretreated for 30 s with 1 μM staurosporine (b), 100 μM DiC₈ (d), and 1 μM PMA (f) were exposed to 5 μM ionomycin in the presence or absence of extracellular Ca\(^{2+}\). Confocal images of PKCα-GFP were obtained before (a, b, d, and f) and 30 s (c, e, and f), 2 min (c, e, and f), 120 s (c, e, and f), and 220 s (a, b, d, and f) after stimulation with 5 μM ionomycin (Iono). Extracellular Ca\(^{2+}\) was removed 180 s after stimulation. In control experiments, images were acquired before (c1 and e1), and 2 (c2 and e2) and 20 min (c3 and e3) after treatment with DiC₈ (c) and PMA (e). Scale bar: 20 μm. The time course of changes in the fluorescence of PKCα-GFP and Fura Red (a5, b5, d5, and f5). Fluorescence of PKCα-GFP and Fura Red are indicated by open symbols and closed circles, respectively. The presence of ionomycin and Ca\(^{2+}\) is indicated by the horizontal bars at the top of the panel. The ionomycin-induced transient translocation of PKCα-GFP was observed in 16 of 18 cells. The results of staurosporine, DiC₈, or PMA in ionomycin-treated and untreated cells are representative of 10–23 cells.
Regulation of PKCα-GFP Translocation

Fig. 6. Ca$^{2+}$-dependent membrane binding of PKCα-GFP in permeabilized cells. PKCα-GFP-expressing cells were exposed to ICM containing 100 μg/ml saponin. a–e, images were obtained at the time indicated in panel f. Scale bar: 20 μm. f, time course of the change in fluorescence (in arbitrary units) of the cell indicated by the arrow in panel a. The presence of saponin and the calculated [Ca$^{2+}$]$_{i}$ are indicated by the horizontal bars. dP indicates the fraction of Ca$^{2+}$-dependent binding. Results are representative of 18 cells.

Effect of DiC8 on the Ca$^{2+}$- and ATP/Mg-dependent Dissociation of PKCα-GFP—The experiments depicted in Figs. 6 and 7 clearly indicate that the effect of Ca$^{2+}$ and PMA on PKCα-GFP membrane binding and the role of kinase activities in facilitating the release are preserved in saponin-permeabilized cells. To further analyze the interplay of Ca$^{2+}$, DiC8, and the kinase activities for the binding of PKCα-GFP to cell membranes, permeabilized cells were exposed to various test media, and the resulting time courses of the release of PKCα-GFP were analyzed quantitatively. As shown in Fig. 9, A and B, the time course conforms well to a single exponential decay (see “Experimental Procedures”). Accordingly, we have used least-squares curve-fitting techniques to determine the initial rate of PKC release, thereby quantitating our results. The calculated initial rates are plotted as a function of [Ca$^{2+}$]$_{i}$ in Fig. 9C.

In the absence of ATP/Mg, the release rate gradually decreased as the [Ca$^{2+}$]$_{i}$ increased (Fig. 9A, a–c), and release was blocked almost completely by 100 or 250 nM [Ca$^{2+}$]$_{i}$ (Fig. 9A, d and e). The addition of DiC8 slowed the release of PKCα-GFP in the presence of 1.5, 25, and 50 nM [Ca$^{2+}$]$_{i}$, (Fig. 9A, a–c, and C). The half-maximal Ca$^{2+}$ concentrations (the concentration required to reduce the release rate by 50%) in the absence and presence of DiC8 were estimated to be 12.1 and 13.7 nM, respectively (Fig. 9C). DiC8 decreased the release rate by ~50%. These results clearly demonstrate that DiC8 attenuates the release of PKCα-GFP without affecting the efficacy of Ca$^{2+}$. Thus, DiC8 and Ca$^{2+}$ appear to act independently in mediating the binding of PKCα-GFP to cell membranes.

In the presence of ATP/Mg, the release rate also decreased with increased [Ca$^{2+}$]$_{i}$ (Fig. 9B, a–c), whereas the Ca$^{2+}$ concentrations required to prevent the release were markedly higher than those required in the absence of ATP/Mg. Half-maximal Ca$^{2+}$ concentrations in the absence and presence of DiC8 were estimated to be 57.9 and 66.8 nM, respectively. Thus, the efficacy of Ca$^{2+}$ in inducing the binding of PKCα-GFP to cell membranes was reduced to ~20% by ATP/Mg. Furthermore, unlike in the presence of ATP/Mg, in the presence of ATP/Mg the effect of DiC8 was altered by [Ca$^{2+}$]$_{i}$. Although DiC8 failed to slow the release of PKCα-GFP in the presence of ATP/Mg and [Ca$^{2+}$]$_{i}$ lower than 500 nM (Fig. 9, B and D), it decreased the release rate to ~50% in the presence of high [Ca$^{2+}$]$_{i}$ (above 1000 nM) and ATP/Mg. This result clearly demonstrates that in the presence of ATP/Mg, DAG selectively potentiates the effect of high concentrations of Ca$^{2+}$ on the membrane binding of PKCα-GFP. It appears, therefore, that in intact cells, this interplay between Ca$^{2+}$, DAG, and ATP/Mg may underlie the DAG-dependent enhancement of the translocation of PKCα-GFP during agonist-induced Ca$^{2+}$ oscillations.

DISCUSSION

We have demonstrated that agonist-induced Ca$^{2+}$ oscillations cause a parallel oscillatory translocation of PKCα-GFP to and from the plasma membrane. This oscillatory translocation of PKCα-GFP is accomplished by an interplay between Ca$^{2+}$ and kinase activities, where the translocation to the membrane is regulated by the increase in [Ca$^{2+}$]$_{i}$, and the relocation to the cytosol is regulated by the kinase activity in addition to the increase in [Ca$^{2+}$]$_{i}$. More strikingly, we found that DAG increases the amplitude of the oscillatory translocation of PKCα by delaying the dissociation of PKCα-GFP, so that the translocation overcomes the relocation. To the best of our knowledge, this is the first evidence for DAG as an amplitude modulator for the oscillatory translocation of ePKCs.

It has been proposed that DAG delays the dissociation of PKCγ and leads to a persistent membrane translocation of
FIG. 9. Quantitative analysis of the effect of Ca\(^{2+}\), DAG, and ATP/Mg on the release of PKC-GFP in permeabilized cells. Permeabilized cells were exposed to various test media at time 0, and the time course of the change in fluorescence was monitored. A, release of PKC-\(\alpha\)-GFP in ICM containing 1.5 (a), 25 (b), 50 (c), 100 (d), and 250 nM (e) Ca\(^{2+}\) in the absence of ATP and MgCl\(_2\) and with (open squares) or without (closed squares) 100 \(\mu\)M DiC8. B, release of PKC-\(\alpha\)-GFP in ICM containing 1.5 (a), 250 (b), 500 (c), 1000 (d), and 3800 nM (e) Ca\(^{2+}\) in addition to 3 mM ATP and 1.4 mM MgCl\(_2\) and with (open circles) or without (closed circles) 100 \(\mu\)M DiC8. Results are shown as mean \pm S.E. of 16–47 cells examined. The curves were drawn by fitting the data to a single exponential equation (see “Experimental Procedures”). C, the initial rates of the fluorescence release were calculated from data shown in A and B (see “Experimental Procedures”) and were plotted as a function of Ca\(^{2+}\) concentration. D, the inhibitory effect of DiC8 on the release of PKC-\(\alpha\)-GFP is expressed as percent inhibition. The percent inhibition was calculated from initial rates as follows: [rate in control − rate with DiC8]/rate in control (solid bars). The percent inhibition was also calculated from data shown in A and B (hatched bars). Ca\(^{2+}\) concentrations and the presence or absence of ATP/Mg are indicated at the bottom of panel D. Results are represented as mean \pm S.E. of 12–47 cells.
PKCγ during high- but not low-frequency Ca^{2+} spikes (6). This proposal points to the possibility that PKCγ serves as a molecular machine for decoding Ca^{2+} oscillations in combination with the DAG signal. However, while we have shown that DAG delayed the dissociation of PKCs after the termination of a Ca^{2+} spike, we did not observe persistent membrane translocation of PKCs during agonist-induced Ca^{2+} oscillations after DAG had been added. The difference between the present and previous studies may be attributable to the different cell lines used or to the different PKC isoforms involved. It is possible that the PKCα and PKCγ isoforms have different functions; for example, PKCα may act as an amplifier, whereas PKCγ may serve as a decoder of Ca^{2+} oscillation.

Regarding the physiological relevance of this amplitude modulation, we postulate that DAG signals play a crucial role in recruiting PKCα to specific sites within the plasma membrane where phosphoinositide metabolism is locally activated. Unlike the rapid diffusion of the resulting Ca^{2+} signals, DAG signals would diffuse more slowly, remaining localized for a longer time in the specific site (25). Indeed, in macrophage cell lines, the generation of local DAG signals (26) and the consequent accumulation of PKCs and PKCδ to the phagosomal membranes have been suggested (27–29). Recent reports using GFP-tagged PKCs have shown that PKCα accumulates in regions of cell-cell contact (20) and at focal spots of the plasma membrane (15). These authors have also speculated that there is a requirement for some other unidentified signal(s) in addition to Ca^{2+}. We anticipate that the spatial and temporal regulation of cPKCs is controlled by the interplay of Ca^{2+}, DAG, and phosphorylation. This type of restricted signaling process to small subregions of the cell might be relevant in polar cells such as epithelial cells, chemotactic cells, and neuronal cells (25).

In experiments using ionomycin, we demonstrated that PKCα-GFP translocates to the plasma membrane in a transient manner despite the continuous elevation of [Ca^{2+}]. Preincubation with staurosporine blocks the relocation of PKCα-GFP to the cytosol during [Ca^{2+}], elevation, indicating that the relocation of PKCα-GFP is dependent on kinase activities. More importantly, it is interesting to note that staurosporine requires [Ca^{2+}], elevation to enhance binding of PKCα-GFP to the plasma membrane and cannot block the relocation of PKCα-GFP to the cytosol after the removal of Ca^{2+}. This suggests that the phosphorylation of PKC or other proteins exerts its effect by modulating the affinity of PKCα-GFP for Ca^{2+}. In agreement with this observation, we found that membrane-bound PKCα-GFP in permeabilized cells was released in ATP/Mg-dependent manner. It has been shown that PKCβII requires autophosphorylation at Thr-641 and Ser-660 to be released from membranes (12, 21). Homologous autophosphorylation sites are also present in PKCε (10, 13). Although membrane-bound PKCα-GFP was phosphorylated, we did not find that phosphorylation of PKCα-GFP increased after ATP/Mg-dependent release. This experiment suggests that, in addition to the autophosphorylation of PKC, the phosphorylation of proteins other than PKCs may be involved in the ATP/Mg-dependent release of membrane-bound PKCα. Additional work will be required to explore this possibility.

It is generally known that the effect of DAG and Ca^{2+} on the translocation of cPKC isoforms are mediated by the C1 and C2 domains, respectively. In addition, a number of aspects of PKC regulation have been linked to their phosphorylation sites (10, 12, 13, 21, 24). Molecular mechanisms involved in the translocation of cPKCs have been extensively examined with mutants that lack phosphorylation site(s) and functional regulatory domain(s). Although these studies provide important findings concerning the function of the regulatory domains and phosphorylation sites, the details of how these domains interact and the role of phosphorylation plays in the membrane translocation of cPKCs remain largely unclear.

To attempt to resolve these issues, we directly examined the effects of Ca^{2+}, DAG, and ATP/Mg on the binding between PKCα-GFP and cell membranes using saponin-permeabilized cells. Because our initial data showed that saponin-permeabilized cells preserved the responsiveness of the translocation of PKCα-GFP to Ca^{2+}, PMA, DiC8, and ATP/Mg (Figs. 6 and 7), the membrane interaction of PKCα-GFP is apparently intact in this permeabilized cell system. We then extended this technique to analyze the process by which PKCα-GFP dissociates from the cell membranes in permeabilized cells (Fig. 9). This simple technique allows us to directly assess the roles PKC regulators and their interactions play in the binding of PKC to biological membranes in close to physiological conditions. We learned the following: 1) DAG supports the binding between PKCα-GFP and membranes regardless of Ca^{2+} concentrations; 2) ATP/Mg decreases the effect of Ca^{2+} on the membrane binding of PKCα-GFP and thereby accelerates the release of PKCα-GFP; 3) ATP/Mg attenuates the DAG-mediated membrane binding of PKCα-GFP at lower Ca^{2+} concentrations (<500 nM); (4) high concentrations of Ca^{2+} (>1000 nM) restore the DAG-mediated membrane binding of PKCα-GFP in the presence of ATP/Mg. These results directly support some of the current hypotheses discussed below and provide new insights into the collaborative regulation of PKCs by Ca^{2+}, DAG, and phosphorylation.

We found that DAG prolongs the Ca^{2+}-mediated binding of PKCα-GFP to the plasma membrane, whereas DAG alone exerts no effect on the translocation of cytosolic PKCα-GFP in intact cells. These observations support the proposition that cPKCs require Ca^{2+}-mediated translocation to become accessible to DAG (6, 30). However, unlike the translocation of cytosolic PKCα-GFP, the release of the membrane-bound PKCα-GFP in permeabilized cells was slowed by DAG regardless of the Ca^{2+} concentration, suggesting that it is the binding of cPKCs to phospholipids, rather than to Ca^{2+}, that makes these enzymes accessible to DAG. In addition, this implies that the Ca^{2+}-binding site and the DAG-binding site of membrane-bound PKCs do not interact directly, as suggested by studies on PKCβII (31, 32). Given that DAG-mediated membrane binding of PKCα-GFP is attenuated by ATP/Mg when the [Ca^{2+}]_m is lower than 500 nM but the DAG-mediated binding is restored in the presence of ATP/Mg with high concentrations of Ca^{2+}, it appears that DAG and phosphorylation collaborate to make this enzyme selectively responsive to high Ca^{2+} concentrations. Although the underlying mechanisms have yet to be clarified, the present study provides the first detailed insight into the way Ca^{2+}, DAG, and phosphorylation concertedly regulate the oscillatory translocation of cPKCs.

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