Decoding of Short-lived Ca\(^{2+}\) Influx Signals into Long Term Substrate Phosphorylation through Activation of Two Distinct Classes of Protein Kinase C\(^*\)\(\S\)

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In electrically excitable cells, membrane depolarization opens voltage-dependent Ca\(^{2+}\) channels eliciting Ca\(^{2+}\) influx, which plays an important role for the activation of protein kinase C (PKC). However, we do not know whether Ca\(^{2+}\) influx alone can activate PKC. The present study was conducted to investigate the Ca\(^{2+}\) influx-induced activation mechanisms for two classes of PKC, conventional PKC (cPKC; PKC\(\alpha\)) and novel PKC (nPKC; PKC\(\theta\)), in insulin-secreting cells. We have demonstrated simultaneous translocation of both DsRed-tagged PKC\(\alpha\) to the plasma membrane and green fluorescent protein (GFP)-tagged myristoylated alanine-rich C kinase substrate to the cytosol as a dual marker of PKC activity in response to depolarization-evoked Ca\(^{2+}\) influx in the DsRed-tagged PKC\(\alpha\) and GFP-tagged myristoylated alanine-rich C kinase substrate co-expressing cells. The result indicates that Ca\(^{2+}\) influx can generate diacylglycerol (DAG), because cPKC is activated by Ca\(^{2+}\) and DAG. We showed this in three different ways by demonstrating: 1) Ca\(^{2+}\) influx-induced translocation of GFP-tagged C1 domain of PKC\(\gamma\), 2) Ca\(^{2+}\) influx-induced translocation of GFP-tagged pleckstrin homology domain, and 3) Ca\(^{2+}\) influx-induced translocation of GFP-tagged PKC\(\theta\), as a marker of DAG production and/or nPKC activity. Thus, Ca\(^{2+}\) influx alone via voltage-dependent Ca\(^{2+}\) channels can generate DAG, thereby activating cPKC and nPKC, whose activation is structurally independent of Ca\(^{2+}\).

Since the first molecular cloning and sequencing of a bovine brain protein kinase C (PKC)\(^1\) (1), PKC has been one of the most extensively studied enzymes in eukaryotic cells. We now know that PKC plays a pivotal role in a myriad of cellular functions. Ten isoforms of PKC have been identified so far and have been classified into three categories based on structural differences in the regulatory domain: conventional PKC (cPKC; PKC\(\alpha\), PKC\(\beta\), PKC\(\delta\), PKC\(\epsilon\), PKC\(\iota\), and PKC\(\gamma\)), novel PKC (nPKC; PKC\(\delta\), PKC\(\epsilon\), PKC\(\iota\), and PKC\(\gamma\)), and atypical PKC (PKC\(\zeta\) and PKC\(\lambda\)) (2, 3). The C1 and C2 regions in the regulatory domain are responsible for diacylglycerol (DAG) and Ca\(^{2+}\) binding, respectively. DAG comes mainly from plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) hydrolysis. This is caused by phospholipase C (PLC) activation, following agonist binding to a G protein-coupled receptor. In excitable cells, cytosolic Ca\(^{2+}\) signals are generated either by Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels (VDCCs) and/or by Ca\(^{2+}\) release from the endoplasmic reticulum through inositol 1,4,5-trisphosphate (IP\(_3\)) receptors upon binding of IP\(_3\), the other product of PIP\(_2\) hydrolysis (4, 5). DAG and Ca\(^{2+}\) activate the first family of cPKCs that have both the C1 and C2 regions. The second family of the novel PKCs is also activated by DAG, but in a Ca\(^{2+}\)-independent manner because of the absence of the functional C2 region. The third family of the atypical PKCs can be activated by phosphoinositide-dependent kinase 1 in a Ca\(^{2+}\)-independent manner (6).

We focused this study on PKC\(\alpha\) and PKC\(\theta\) as representatives of cPKC and nPKC, respectively, to probe further into the mechanisms underlying the Ca\(^{2+}\) signaling-induced activation of cPKC and nPKC. To this end, we employed INS-1 cells, an insulin-secreting cell line established from a rat insulinoma (7), as a model system in which VDCCs are the main pathways for the generation of Ca\(^{2+}\) signals. Key observations from other laboratories have shown that PKC\(\alpha\) is activated within the physiological Ca\(^{2+}\) concentration range in the presence of both DAG and phosphatidylyserine (8) and that depolarization K\(^+\) concentrations evoke an increase in the IP\(_3\) concentration in rat pancreatic islets, suggesting PLC-mediated production of DAG (9). Two important questions arise: 1) Can depolarization-evoked Ca\(^{2+}\) influx through the opening of VDCCs activate cPKC? and 2) Can Ca\(^{2+}\) influx also activate nPKC, if the Ca\(^{2+}\) dependent Ca\(^{2+}\) channel; IP\(_3\), inositol 1,4,5-trisphosphate; GFP, green fluorescent protein; MARCKS, myristoylated alanine-rich C kinase substrate; -GFP, GFP-tagged; -DsRed, DsRed-tagged; PHD, pleckstrin homology domain; TPA, 12-O-tetradecanoylphorbol-13-acetate; DiC\(_8\), 1,2-dioctanoyl-sn-glycerol; TRITC, tetramethylrhodamine isothiocyanate; TIRFM, total internal reflection fluorescence microscopy; TEA, tetraethylammonium; Ach, acetylcholine; ER, endoplasmic reticulum.
influx results in production of DAG? Furthermore, in INS-1 cells, as in normal insulin-secreting cells, glucose-induced oscillations in membrane potential elicit repetitive openings of VDCCs (10). This is responsible for oscillations in the cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]i), which causes pulsatile insulin secretion (11). Ca$^{2+}$ oscillations play an essential role in exocytotic secretion in neuroendocrine cells (12). It is therefore important to know how depolarization-evoked Ca$^{2+}$ oscillations are decoded into long term physiological modifications, such as insulin secretion, through PKC activation.

Recent advances in the use of green fluorescence protein (GFP) have allowed us to investigate PKC activity in intact living cells by monitoring translocation of GFP-tagged PKC (13, 14). Inactive PKCs are located in the cytosol. Upon activation, following PIP$_2$ hydrolysis, they translocate from the cytosol to other cellular locations, such as the plasma membrane. By simultaneously measuring the cytosolic Ca$^{2+}$ concentration and the translocation of GFP-PKC in astrocytes, it has been shown that there is a marked temporal correlation between glutamate-elicited Ca$^{2+}$ spikes and PKC translocation (15). A current model for activation of cPKC (14) proposes that: 1) the [Ca$^{2+}$i], recruitment cPKC to the plasma membrane via the C2 region, 2) the site on the enzyme where the pseudosubstrate inhibitory region in the regulatory domain is occupied at the site is exposed and becomes available for substrate binding, and 3) full activation of the enzyme takes place when DAG tightly tethers the enzyme to the plasma membrane via the C1 region. The sequence of these events suggests that translocation and activation of cPKC may not always correspond. This raises a final question: How do we know when the pseudosubstrate inhibitory region is removed (i.e., when exactly does activation of cPKC take place)?

To address these questions, we monitored translocation of PKCa-GFP, as markers for cPKC, in response to depolarization-evoked Ca$^{2+}$ influx through VDCCs in INS-1 cells. The Ca$^{2+}$ influx resulted in translocation of PKCa-GFP to the plasma membrane. We also assessed the phosphorylation state of the PKC substrate myristoylated alanine-rich C kinase substrate (MARCKS) (16) as another marker of PKC activity, by monitoring translocation of GFP-tagged MARCKS (MARCKS-GFP) with DeRed-tagged PKCa (PKCa-DeRed). When phosphorylated by PKC, MARCKS translocates from the plasma membrane to the cytosol (17). Translocation of MARCKS-GFP to the cytosol took place as soon as PKCa-DeRed translocated to the plasma membrane upon stimulation of Ca$^{2+}$ influx. These results indicate that the Ca$^{2+}$ influx can generate DAG, because cPKC is activated by Ca$^{2+}$ and DAG. We showed this in three different ways by demonstrating: 1) Ca$^{2+}$ influx-induced translocation of GFP-tagged C1 domain of PKCy, 2) Ca$^{2+}$ influx-induced translocation of GFP tagged pleckstrin homology domain (GFP-PHD), and 3) Ca$^{2+}$ influx-induced translocation of PKCp-GFP as a marker of DAG production. The depolarization-evoked increase in DAG concentration was estimated from in situ calibration to be 1.90 ± 0.02 μM. We have demonstrated for the first time that depolarization-evoked Ca$^{2+}$ influx can generate DAG, thereby activating cPKC and nPKC. We also observed that MARCKS remained phosphorylated through PKC activation as long as the depolarization-evoked Ca$^{2+}$ oscillations continued. Our results show that short-lived Ca$^{2+}$ signals can be transduced via PKC activation into long term phosphorylated MARCKS.

**Experimental Procedures**

**Plasmid Construction**

PKCa-pEFGP, PKCp-pEFGP, pEGFP-N2, and pDeRed1-N1 were obtained from Clontech Lab, Inc. (Palo Alto, CA). To attain brighter fluorescence of MARCKS-GFP, the GFP of MARCKS-GFP (17) was replaced with pEGFP-N2. pEGFP of PKCa-pEGFP was replaced with pDsRed1-N1. A GFP-tagged C1 region of PKCy (C1-GFP) was produced from a DNA clone of λKBrY1, which was subcloned into an expression plasmid for mammalian cells, pTB701 (18). A cDNA fragment of PKCy for C1 region with an EcoRI site in the 5’ terminus and a BglII in the 3’ terminus was produced by PCR using pTB701 as a template. The sense and antisense primers used were 5’-TTGAAT- TCCCAGCTGTTAACGGCAG-3’ and 5’-TTGATCTGTCCAGCCGGAGGGAGG-3’, respectively. A PCR product for C1 region of PKCy was subcloned into the EcoRI site and the BglII site in GFP containing pTB701 (18). The PCR product was verified by sequencing. A GFP-tagged pleckstrin homology domain of PLCδ1 (GFP-PHD) was donated by Dr. Hirose (Tokyo University, Tokyo, Japan) (19).

**Cell Culture and Transfection**

INS-1 cells (7), insulin-producing cells, were a gift from Dr. Sekine (Tokyo University). The cells were grown in 100-mm culture dishes at 37 °C and 5% CO$_2$ in a humidified atmosphere. The culture medium was RPMI 1640 with 10% fetal bovine serum, 1 mM sodium pyruvate, and 50 μM mercaptoethanol. For fluorescence imaging, the cells were cultivated on a coverslip at 50% confluency 2 days before transfection. A plasmid of the GFP- or DeRed-tagged proteins was transfected into the cells by lipofection using TransIT™-LT1 (Mirus, Madison, WI). The experiments were performed 2 days after transient transfection.

**Solutions**

The standard extracellular solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 2.5 mM CaCl$_2$, 3 mM glucose, and 10 mM Hepes-NaOH (pH 7.3). The solutions for membrane depolarization contained 165 mM NaCl, 40 mM KCl, 1 mM MgCl$_2$, 2.5 mM CaCl$_2$, 3 mM glucose, and 10 mM Hepes-NaOH (pH 7.3) or contained 120 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 2.5 mM CaCl$_2$, 20 mM tetraethylammonium chloride, 3 mM glucose, and 10 mM Hepes-NaOH (pH 7.3). In some experiments, CaCl$_2$ was not included (Ca$^{2+}$-free solution). The cells, placed on a glass coverslip attached to an open perfusion chamber, were washed for 15 min from Calbiochem (La Jolla, CA).

**Imaging Experiments**

**Epifluorescence Microscopy**—The fluorescence images were captured using a Olympus inverted microscope (40×, water immersion objective, and 60×) equipped with a cooled -50°C coupled charge device digital camera (ORCA-II and ORCA-ER, Hamamatsu Photonics, Hamamatsu, Japan) and recorded and analyzed on an Aquacosmos imaging station (Hamamatsu Photonics). The excitation light source was a 150 W xenon lamp with a Polychrome I monochromator (T.I.L.L. Photonics GmbH, Planegg, Germany). GFP fluorescence was excited at 488 nm for high time resolution of GFP-tagged PKCs digital imaging. We measured the fluorescence intensity of the GFP (DeRed)-tagged proteins (PKCs, MARCKs, PHD, and C1 domain) in the cytosol of the cell, excluding the nucleus, and/or at the plasma membrane, as a marker of translocation. These values (F) were normalized to each initial value (F0), so that the relative fluorescence change was referred to as the “ratio F/F0.” For simultaneous measurements of the relative change in fluorescence intensity of the GFP-tagged PKCs in the cytosol and [Ca$^{2+}$]i, GFP fluorescence was excited at 488 nm, whereas Fura2 was excited at wavelengths alternating between 340 and 380 nm. We put a short pass filter of 330–385 nm to reduce background fluorescence in the light pass between a dichroic mirror of 505 nm and an emission filter of 525/45 nm band pass. The cells transfected expressing these transgenes were loaded with 2 μM Fura2/AM in the standard extracellular solution for 30 min at room temperature. The cells were washed twice and used within 2 h. The Fura2 ratio was calibrated using exposure to 10 μM ionomycin and 10 mM Ca$^{2+}$ or 10 mM EGTA in the Fura2-loaded cells without transfection of the GFP-tagged PKCs. A dissociation constant of 12 μM for Ca$^{2+}$ and Fura2 at 37 °C was used. For simultaneous measurement of relative fluorescence change in intensity of MARCKS-GFP and PKCa-DeRed using a dual band for fluorescein isothiocyanate and TRITC, GFP fluorescence was excited at 488 nm, whereas PKCa-DeRed fluorescence was excited at 558 nm. To reduce
cross-talk between them, an emission filter wheel was used, and alternate emission filters of 535/45-nm band pass and 605/50-nm long pass were synchronously set with excitation filters for GFP and DsRed.

TIRFM (Evanescent Wave)—To obtain high signal-to-noise ratio over the conventional epifluorescence microscopy (see supplemental figure), we installed a TIRFM unit (Olympus) into the same imaging system mentioned above. The incidental light was introduced from the objective lens for TIRFM (Olympus NA = 1.45, 60×). GFP and Fura Red were excited by a 488-nm laser, and each emitted light was collected through 535/45 and 605/50 nm, respectively. For simultaneous measurement of relative fluorescence change in intensity of PKC-GFP and [Ca$^{2+}$]$_i$, in the Fura Red-loaded cells, we used a W-view Optics (Hamamatsu Photonics), a branching optics that splits the incident light into using a Dichroic mirror of 550 nm, so that two separate images of the GFP and Fura Red fluorescence can be produced.

RESULTS

Membrane Depolarization Induces Transient Translocation of PKCa—We first examined the distribution of PKC-GFP with the help of high time resolution digital imaging. Fig. 1A shows the rapid and reversible translocation of PKC-GFP in response to a depolarizing K$^+$ concentration (40 mM), which evoked Ca$^{2+}$ influx through opening of VDCCs. The relative changes in the fluorescence intensities of PKC-GFP in the cytosol and at the plasma membrane are plotted in Fig. 1B, as a function of time. PKC-GFP was translocated from the cytosol to the plasma membrane, and this can be seen by the reciprocal changes in the two parameters (Fig. 1B) (n = 6). Thus, either parameter can be used as a marker of PKC-GFP plasma membrane translocation. We chose to employ the relative fluorescence change in the cytosol as a marker of translocation. Next, we simultaneously measured [Ca$^{2+}$]$_i$ and PKC translocation in Fura2-loaded and PKC-GFP-expressing INS-1 cells. As seen in Fig. 1C, a depolarizing K$^+$ concentration induced a transient translocation of PKC-GFP to the plasma membrane following a transient [Ca$^{2+}$]$_i$ elevation (n = 8). Thus, the temporal profile was similar to that observed when PKC-GFP measurements were carried out alone, suggesting successful dissection of Ca$^{2+}$ and GFP signals.

Threshold Value of [Ca$^{2+}$]$_i$ for PKCα Translocation—We loaded INS-1 cells with Fura2, without expressing PKC-GFP, to accurately estimate [Ca$^{2+}$]$_i$. In the standard extracellular
solution containing 2.5 mM CaCl₂ and 3 mM glucose, more than 50% of the Fura2-loaded INS-1 cells displayed spontaneous cytosolic Ca²⁺ oscillations (20). The peak [Ca²⁺], was no more than 400 nM. When the same cells were depolarized by the K⁺ channel blocker tetraethylammonium (TEA) (20 mM), the Ca²⁺ oscillations became more pronounced (21), and the peak [Ca²⁺], was in the range of 600–800 nM (Fig. 2A) (n = 10). To avoid cross-talk between Ca²⁺ and GFP signals, exactly the same protocol as in Fig. 2A was applied to PKCa-GFP-expressing cells without Fura2 loading. No translocation of PKCa-GFP took place in the standard extracellular solution, whereas oscillatory translocations of PKCa-GFP started immediately after introduction of the TEA-containing solution (Fig. 2B) (n = 5), suggesting a threshold value of [Ca²⁺], of more than 400 nM for PKCa translocation. The temporal profile of the TEA-evoked PKCa-GFP translocations (Fig. 2, C and D) (n = 5) was similar to that observed in response to the membrane depolarization evoked by a high K⁺ concentration (Fig. 1B).

Depolarization-evoked Translocation of PKCa Depends on Ca²⁺ Influx but Not on Ca²⁺ Mobilization—Fig. 3A shows that upon removal of external Ca²⁺, the TEA-induced Ca²⁺ oscillations and the PKCa-GFP translocations were abolished (n = 5), indicating that both are totally dependent on Ca²⁺ influx. We then tested whether PKCa can be fully activated in the presence of DAG at physiological Ca²⁺ concentrations. Using PKCa-GFP translocation to the plasma membrane as a marker of activation, short exposure to a combination of TEA and the diacylglycerol analogue DiC₈ (100 μM) induced sustained PKCa activation, despite the fact that [Ca²⁺], quickly returned to the steady resting level upon removal of the stimulation (Fig. 3B) (n = 8). This suggests that even a single TEA-evoked Ca²⁺ spike (within the physiological Ca²⁺ concentration range) can fully activate PKCa in the presence of a sufficient amount of DAG (8).

To compare the effects of Ca²⁺ influx and IP₃-mediated Ca²⁺ mobilization on PKCa-GFP translocation, we tested, in the same cells, the actions of TEA and acetylcholine (ACh) (using a supramaximal concentration (100 μM), which might produce sufficient DAG to activate PKCa (see Figs. 1B and 3A)). As shown in Fig. 4A, the TEA-evoked translocation of PKCa-GFP was much more substantial than that induced by ACh, although the bulk [Ca²⁺], elevations produced by the two agents were nearly equal (n = 11). For more accurate evaluation of the cytosolic Ca²⁺ elevation caused specifically by IP₃-induced store release, we removed the Ca²⁺ influx component due to capacitative Ca²⁺ entry through store-operated Ca²⁺ channels in the plasma membrane (22, 23). This was simply done by removing external Ca²⁺ during stimulation with ACh (100 μM) (Fig. 4B). The result was similar (n = 5) to that shown in Fig. 4A, suggesting that there was little effect of store-operated Ca²⁺ influx on PKCa translocation. Reversing the sequence of events (applying TEA first and then subsequently ACh) gave similar results to those shown in Fig. 4 (data not shown).

PKCa, Activated by Depolarization-evoked Ca²⁺ Influx, Can Phosphorylate Its Substrate, MARCKS—One line of evidence...
has shown that [Ca\textsuperscript{2+}] elevations drive translocation of cPKC to the plasma membrane (13, 14). However, we do not know whether cPKC can be activated by depolarization-evoked Ca\textsuperscript{2+} influx alone. To answer this question, we employed a GFP-tagged MARCKS as another marker of PKC activity (17), which is a putative and direct substrate for PKC (16), as well as PKCα-DsRed. We co-transfected both of them into INS-1 cells. When activated PKC phosphorylates the plasma membrane–anchored MARCKS, then phosphorylated MARCKS translocates from the plasma membrane to the cytosol. Thus, simultaneous monitoring of PKCα-DsRed and MARCKS-GFP allows us to test whether depolarization-evoked Ca\textsuperscript{2+} influx can activate PKCα. Fig. 5 (A and B) shows translocations of MARCKS-GFP and PKCα-DesRed induced by TEA, indicating that depolarization-evoked Ca\textsuperscript{2+} influx can activate PKCα. Phosphorylated MARCKS only slowly and gradually returned to the plasma membrane (≈2.5 min) in contrast to the rapid temporal profile of PKCα translocation (≈30 s) (Fig. 5B) (n = 6). Ca\textsuperscript{2+} oscillation-driven translocations of PKCα kept MARCKS phosphorylated in the cytosol until termination of the repetitive PKCα translocations (Fig. 5C) (n = 8).

Depolarization-evoked Ca\textsuperscript{2+} Influx Induces Translocation of PKCα, Despite the Absence of the Functional C2 Domain for Ca\textsuperscript{2+} Binding—As seen in Fig. 5 (A and B), now we know that depolarization-evoked Ca\textsuperscript{2+} influx can activate PKCα. This observation prompted us to explore whether depolarization-evoked Ca\textsuperscript{2+} influx can generate DAG, because Ca\textsuperscript{2+} and DAG are required for activation of cPKC (2). It has been shown that K\textsuperscript{-}induced membrane depolarization increases IP\textsubscript{3} production in insulin-secreting rat pancreatic islets (9). Taken together, these observations indicate that there should be production of DAG in response to depolarization-evoked Ca\textsuperscript{2+} influx. To test this hypothesis, we employed PKCα-GFP as a marker of nPKC activity as well as DAG production because nPKC is activated by DAG alone in a Ca\textsuperscript{2+}-independent manner (2). TPA caused a rapid and sustained translocation of PKCα-GFP in the complete absence of Ca\textsuperscript{2+} influx (Fig. 6, A and B) (n = 6). The
simultaneous measurements of PKCβ-GFP translocation and cytosolic Ca\(^{2+}\) concentration, shown in Fig. 6C, confirm that extracellular Ca\(^{2+}\) is not required for PKCβ-GFP translocation \((n = 4)\). We then applied a depolarizing K\(^+\) concentration to PKCβ-GFP-expressing cells. Fig. 7A clearly shows that this stimulus induced a gradual translocation of PKCβ-GFP to the plasma membrane, which was reversible upon removal of the high K\(^+\) solution \((n = 12)\). The amplitude of the translocation induced by K\(^+\)-induced depolarization was comparable with that elicited by ACh (Fig. 7B). It should be noted that ACh still continued to induce translocation of PKCβ-GFP after [Ca\(^{2+}\)] \(_i\), had returned to the resting level, verifying that 100 \(\mu\)M ACh generated enough DAG to sustain the translocation until termination of the stimulus \((n = 8)\) (Figs. 7B and 9B). The amplitude of the TEA-evoked translocation was also comparable with that induced by ACh, and the translocation was not synchronous with the TEA-evoked Ca\(^{2+}\) spikes (Fig. 7C) \((n = 8)\).

**Depolarization-evoked Ca\(^{2+}\) Influx Translocates GFP-tagged Pleckstrin Homology Domain of PLC\(\gamma\) (GFP-PHD) and GFP-tagged C1 Domain of PKC\(\gamma\) (C1\(_2\)-GFP)—** To fully corroborate the above evidence that the Ca\(^{2+}\) influx generates DAG and activates PKC\(\theta\), we performed further experiments using GFP-PHD and C1\(_2\)-GFP. First, GFP-PHD allows us to visualize IP\(_3\) production by translocating from the plasma membrane to the cytosol because of the 20-fold higher affinity for IP\(_3\) than for PIP\(_2\) (19), such that we can assess indirectly the simultaneous production of DAG upon PIP\(_2\) hydrolysis by a Ca\(^{2+}\)-dependent PLC activation. As shown in Fig. 8A, depolarization-evoked Ca\(^{2+}\) influx resulted in the relatively transient translocation of PHD-GFP, whereas the translocation was sustained during ACh stimulation \((n = 11)\), indicating DAG production. Second, to directly monitor the plasma membrane DAG levels, we also employed translocation of the C1\(_2\)-GFP as a DAG sensor \((14, 15)\) using TIRFM. This has a ~10-fold higher signal-to-noise ratio (see “Experimental Procedures”) than conventional epifluorescence microscopy in terms of the fluorescent protein translocation near the plasma membrane (for example PKC-GFPs) \((15)\). We loaded the C1\(_2\)-GFP-expressing cells with Fura Red to define the relationship between the [Ca\(^{2+}\)] \(_i\), change and DAG production in response to the Ca\(^{2+}\) influx. Depolarization-evoked Ca\(^{2+}\) influx clearly translocated C1\(_2\)-GFP to the membrane (Fig. 9A) after [Ca\(^{2+}\)] \(_i\), had risen to the peak (Fig. 9B), in contrast to the sustained ACh-induced translocation of C1\(_2\)-GFP following the transient [Ca\(^{2+}\)] \(_i\), elevation. Stimulation with 40 mM K\(^+\) also resulted in a C1\(_2\) translocation of an undiminished magnitude in 5 \(\mu\)M thapsigargin-pretreated C1\(_2\)-GFP-expressing cells, indicating little contribution of ei-
Fig. 9. Depolarization-evoked Ca\textsuperscript{2+} influx and ACh induce C1\textsubscript{2}-GFP translocation. Shown are simultaneous measurements of [Ca\textsuperscript{2+}], and C1\textsubscript{2}-GFP translocation in Fura Red-loaded and C1\textsubscript{2}-GFP expressing INS-1 cells using TIRFM. The ratio is set to 1 at the beginning of each experiment. A, ACh-released C1\textsubscript{2}-GFP remained at the plasma membrane. The translocation was inhibition by Ca\textsuperscript{2+}-free extracellular solution containing 0.2 mM EGTA for the first 2 min followed by consecutive applications of DiC\textsubscript{8} 3 μM (0.35 μg/ml), 10 μM (1.05 μg/ml), and 10 μM (3.5 μg/ml). B, panels a–e were taken at the times indicated by the arrows in A. The region of interest represented by the dashed box was at the plasma membrane. The ratio represents relative fluorescence intensity of C1\textsubscript{2}-GFP. The bar represents 10 μm.

DISCUSSION

Microdomains of Elevated [Ca\textsuperscript{2+}] beneath the Plasma Membrane, but Not Elevation of the Bulk [Ca\textsuperscript{2+}], Are Required for cPKC Translocation—We have shown that there is a threshold value of the bulk [Ca\textsuperscript{2+}], at -400 nM for PKC\textalpha translocation in the insulin-secreting INS-1 cells (Fig. 2A), which is consistent with data from other laboratories (24). More importantly, we have also demonstrated that Ca\textsuperscript{2+} influx is a much stronger stimulus for PKC\textalpha translocation than Ca\textsuperscript{2+} mobilization from intracellular stores, even when the amplitudes of the induced bulk [Ca\textsuperscript{2+}] are similar (Fig. 4A). This finding suggests that microdomains of elevated [Ca\textsuperscript{2+}] beneath the plasma membrane ([Ca\textsuperscript{2+}]\textsubscript{loc}) generated by Ca\textsuperscript{2+} influx through VDCCs may play a pivotal role in cPKC translocation in excitable cells rather than the elevated bulk [Ca\textsuperscript{2+}], which is also consistent with a recent report (25). In other words, there is a threshold value of [Ca\textsuperscript{2+}]\textsubscript{loc} for translocation of cPKC. In neuroendocrine cells, the estimated value of [Ca\textsuperscript{2+}]\textsubscript{loc} at the mouth of open VDCCs is several micromoles/liter (12, 26). This indicates that a local [Ca\textsuperscript{2+}] of several micromole/liter may be required for cPKC translocation.

Ca\textsuperscript{2+} mobilization induced by ACh most likely fails to translocate PKC\textalpha because the [Ca\textsuperscript{2+}] rise at the critical sites is insufficient. This could be due to the distance between the plasma membrane and the IP\textsubscript{3} channels in the ER, combined with the substantial Ca\textsuperscript{2+} buffering capacity in the cytosol (27).
However, it could be argued that Ca\textsuperscript{2+} mobilization from the ER should result in store-operated Ca\textsuperscript{2+} entry (22, 23) and that ACh stimulation therefore also could be expected to cause local Ca\textsuperscript{2+} elevation beneath the plasma membrane. Nevertheless, it would appear (Fig. 4B) that the magnitude of Ca\textsuperscript{2+} influx through store-operated Ca\textsuperscript{2+} channels is insufficient to generate the threshold level of [Ca\textsuperscript{2+}]	extsubscript{ER} needed. Because the entry sites from store-operated Ca\textsuperscript{2+} channels would be very close to Ca\textsuperscript{2+} uptake sites into the ER through the powerful Ca\textsuperscript{2+} ATPase pumps (23, 28–31), the net delivery of Ca\textsuperscript{2+} to the cytosol through store-operated Ca\textsuperscript{2+} channels may be less than through voltage-gated channels even if both channels have similar ranges of Ca\textsuperscript{2+} concentrations at the mouths of their pores. They could also possibly be separately located. Our finding that Ca\textsuperscript{2+} entry through VDCCs is sufficient to cause cPKC and nPKC translocation may be important in relation to the control of glucose-elicited insulin secretion, because it has been shown that the L-type VDCCs and the insulin-containing secretory granules are co-localized (26). Thus, local Ca\textsuperscript{2+} entry in the secretory domains could induce PKC activation important for stimulation of exocytosis (25).

Ca\textsuperscript{2+} Influx through VDCCs Is Both Necessary and Sufficient for Activation of cPKC—To fully substantiate that Ca\textsuperscript{2+} influx through VDCCs is both necessary and sufficient for activation of cPKC, we employed the phosphorylation state of MARCKS as another marker of PKC activity. As seen in Fig. 5B, plasma membrane-anchored MARCKS is turned into phosphorylated MARCKS, thereby moving into the cytosol, as soon as PKC\textalpha

translocated to the plasma membrane by Ca\textsuperscript{2+} influx through VDCCs. In INS-1 cells, in which PKC\textalpha

and PKC\beta

are predominantly expressed (32), endogenous cPKC and nPKC may move to the plasma membrane in the same manner as the exogenous examples. Thus, we have provided the first direct evidence showing that single Ca\textsuperscript{2+} spike-driven translocations of cPKC, whose duration is just 30 s long, enable MARCKS to be phosphorylated. The pseudosubstrate inhibitory region of cPKC has been already removed before the association with MARCKS. The cessation of MARCKS translocation and the return to the prestimulation level is very much slower than that of the cPKC translocation, because of sustained MARCKS phosphorylation. We do not know the exact mechanism, but it might result from the net effect of several factors such as diacylglycerol kinase (17), PKC, or a phosphatase that dephosphorylates MARCKS.

Depolarization-evoked Ca\textsuperscript{2+} Influx through VDCCs Can Generate DAG and Thereby Activate cPKC and nPKC, Whose Activation Is Structurally Independent of Ca\textsuperscript{2+}—nPKC, which lacks the functional C2 domain for Ca\textsuperscript{2+} binding, is activated either by DAG or TPA. This can be seen by the sustained PKC\theta

translocation induced by TPA in the absence of external Ca\textsuperscript{2+} (Fig. 6B). However, depolarization-evoked Ca\textsuperscript{2+} influx through VDCCs can also induce gradual and continuous nPKC translocation to the plasma membrane during [Ca\textsuperscript{2+}]	extsubscript{cyt}, elevation (Fig. 7A). It is possible that some regions of PKC\theta

other than the C1 domain can be associated with the plasma membrane. However, two additional experiments using GPP-PH-D and C1\textgamma-GFP have added further credence to the observations (Fig. 7). First, the Ca\textsuperscript{2+} influx-evoked translocation of GPP-PH-D (Fig. 8A) indicates that DAG can be generated upon PIP\textsubscript{2} hydrolysis mediated by a Ca\textsuperscript{2+}-dependent PLC activation (33), although the amplitude of the PHD translocation may parallel the concentration not of DAG but of IP\textsubscript{3} (19). The translocation of GPP-PH-D induced by depolarization was relatively transient, whereas it was sustained during ACh stimulation. This suggests a relatively transient increase in DAG concentration (3). Second, the Ca\textsuperscript{2+} influx-evoked translocation of C1\textgamma-GFP (Fig. 9A) as a DAG sensor, directly supports the view that DAG synthesis is induced by depolarization-evoked Ca\textsuperscript{2+} influx through VDCCs. The simplest explanation for this surprising observation could be that the Ca\textsuperscript{2+} influx can initiate DAG generation, by triggering PLC activation, thereby translocating and activating nPKC. The fact that the translocation of the C1 domain did not start until the [Ca\textsuperscript{2+}]	extsubscript{cyt} had nearly reached its peak (Fig. 9B), taken together with the observation that sustained Ca\textsuperscript{2+} influx induced by 1 \muM ionomycin kept C1\textgamma-GFP at the plasma membrane (data not shown), suggests that there may be a threshold value of [Ca\textsuperscript{2+}]	extsubscript{cyt} for DAG synthesis. Conversely, DAG synthesis can be detected by monitoring the C1 domain translocation, if the amplitude of the C1 domain translocation parallels the amount of DAG synthesis. Therefore, we tried to estimate the increase in DAG content with in situ calibration (Fig. 10A), which gave a value of 1.90 ± 0.02 \muM (mean ± S.D., n = 3). In a report from another laboratory, using a biochemical assay, it was calculated that the amount of accumulated DAG is 13 pmol/10\textsuperscript{6} cells at 30 s in platelet-derived growth factor-stimulated Balb/c/3T3 cells (34). Given a cell volume of ~1 pl, the DAG concentration would be 13 \muM, which is comparable with our data. As shown in Fig. 5, a DAG concentration of ~2 \muM, induced by depolarization-evoked Ca\textsuperscript{2+} influx, may be sufficient to ensure that activated PKC can phosphorylate MARCKS. Monitoring of C1\textgamma-GFP translocation has been the most sensitive way of detecting DAG synthesis beneath the plasma membrane so far (14, 15). As shown in Fig. 9A, the C1 domain bипhiscally translocated to the membrane during high K\textsuperscript{+} stimulation; the first phase was transient, and the second phase was sustained. This indicates continuous production of DAG. DAG synthesis can be mediated by PLC (Figs. 8A and 9D) (33) and/or phospholipase D activated by the [Ca\textsuperscript{2+}]	extsubscript{ER}, rise and/or PKCs (3, 9, 35, 36). However, neither Ca\textsuperscript{2+}—nor IP\textsubscript{3}-induced Ca\textsuperscript{2+} mobilization from the Ca\textsuperscript{2+} stores is important for DAG synthesis, because of the undiminished magnitude of the depolarization-evoked C1\textgamma translocation in the thapsigargin-treated cells.

Our data also have important implications for cPKC activation. If the amplitude of the nPKC translocation reflects the amount of DAG synthesized, depolarization-evoked Ca\textsuperscript{2+} influx could translocate as well as activate cPKC by generating [Ca\textsuperscript{2+}]	extsubscript{cyt} and DAG. Ca\textsuperscript{2+} signals per se, such as action potential-induced Ca\textsuperscript{2+} oscillations, could function as second messengers as well as operate as primary activators of cPKC and nPKC in neuronal, endocrine and muscle cells. In other words, Ca\textsuperscript{2+} signals and the two PKCs signals may not be segregated in certain conditions. Therefore, the roles of these signals in a myriad of cellular functions may overlap.

Short-lived Ca\textsuperscript{2+} Signals through PKC Activation Are Transduced into Long-lived Phosphorylated MARCKS; Ca\textsuperscript{2+} Oscillation-driven Activation of cPKC and nPKC May Modulate Long Term Physiological Phenomena—Our finding that Ca\textsuperscript{2+} oscillation-evoked activation of both PKC and nPKC can keep MARCKS phosphorylated (Fig. 5) has important implications for the control of long term physiological phenomena such as insulin secretion (37), long term potentiation (38), the redox state in the mitochondria (39), and the control of gene expression (40, 41). We can envisage that, as long as Ca\textsuperscript{2+} oscillation-driven activation of a first kinase such as PKC continues in a “sinus-like” manner, then the first kinase is maintained in the phosphorylated state, which in turn leads to activation of a second kinase on a time scale of hours or days. In this way, not only Ca\textsuperscript{2+} oscillations but also Ca\textsuperscript{2+} oscillation-driven activation of both PKCs may modulate a long term physiological phenomenon. Thus, we should bear in mind that two classes of PKCs can be activated in conditions where Ca\textsuperscript{2+} oscillations take place.
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Decoding of Short-lived Ca\textsuperscript{2+} Influx Signals into Long Term Substrate Phosphorylation through Activation of Two Distinct Classes of Protein Kinase C

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