Distinct Effects of Fatty Acids on Translocation of γ- and ε-Subspecies of Protein Kinase C

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Abstract. Effects of fatty acids on translocation of the γ- and ε-subspecies of protein kinase C (PKC) in living cells were investigated using their proteins fused with green fluorescent protein (GFP). γ-PKC–GFP and ε-PKC–GFP predominated in the cytoplasm, but only a small amount of γ-PKC–GFP was found in the nucleus. Except at a high concentration of linoleic acid, all the fatty acids examined induced the translocation of γ-PKC–GFP from the cytoplasm to the plasma membrane within 30 s with a return to the cytoplasm in 3 min, but they had no effect on γ-PKC–GFP in the nucleus. Arachidonic and linoleic acids induced slow translocation of ε-PKC–GFP from the cytoplasm to the perinuclear region, whereas the other fatty acids (except for palmitic acid) induced rapid translocation to the plasma membrane. The target site of the slower translocation of ε-PKC–GFP by arachidonic acid was identified as the Golgi network. The critical concentration of fatty acid that induced translocation varied among the 11 fatty acids tested. In general, a higher concentration was required to induce the translocation of ε-PKC–GFP than that of γ-PKC–GFP, the exceptions being tridecanoic acid, linoleic acid, and arachidonic acid. Furthermore, arachidonic acid and the diacylglycerol analogue (DiC8) had synergistic effects on the translocation of γ-PKC–GFP. Simultaneous application of arachidonic acid (25 μM) and DiC8 (10 μM) elicited a slow, irreversible translocation of γ-PKC–GFP from the cytoplasm to the plasma membrane after rapid, reversible translocation, but a single application of arachidonic acid or DiC8 at the same concentration induced no translocation.

These findings confirm the involvement of fatty acids in the translocation of γ- and ε-PKC, and they also indicate that each subspecies has a specific targeting mechanism that depends on the extracellular signals and that a combination of intracellular activators alters the target site of PKCs.

Key words: protein kinase C • translocation • fatty acid • targeting • green fluorescent protein

PROTEIN kinase C (PKC)[1] is a family of serine/threonine protein kinases that is activated in the presence of phospholipid and Ca^{2+} ions (Nishizuka, 1984, 1988; Parker and Dekker, 1997; Srinivasan and Blundell, 1997). To date, at least 12 subspecies have been identified, and these are divided into three groups based on their structures: conventional, novel, and atypical PKC (Nishizuka, 1988, 1992). The PKC family comprises the regulatory domain in the amino terminus and a catalytic domain in the carboxyl terminus. The conventional PKCs (cPKC), including α-, βI-, βII-, and γ-PKC, have two common regions (C1 and C2) in the regulatory domain. The C1 region has two cysteine-rich loops (zinc finger–like motifs) that are the binding site for diacylglycerol (DAG) and phorbol ester (Nishizuka, 1988; Ono et al., 1989). The C2 region binds to calcium (Ono et al., 1989). The novel PKCs (nPKC), δ-, ε-, η-, and θ-PKC, lack the C2 region (Ono et al., 1988*b*; Hug and Sarre, 1993; Osada et al., 1992). The atypical PKCs (aPKC), ζ- and ι/λ-PKC, lack the C2 region and have only one cysteine-rich loop in the C1 region (Ono et al., 1989; Selbie et al., 1993; Akimoto et al., 1994). Calcium, phosphatidylserine, and DAG are required for the activation of cPKCs, whereas calcium is not required for the activation of nPKCs. aPKCs are insensitive to both DAG and calcium (Newton, 1997).

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1. *Abbreviations used in this paper*: cPKC, nPKC, and aPKC, conventional, novel, and atypical PKCs; DAG, diacylglycerol; DiC8, 1,2-dioctanoylgrecerol; DO, diolein; GFP, green fluorescent protein; PKC, protein kinase C; PS, phosphatidylserine; TPA, 12-*o*-tetradecanoylphorbol 13-acetate.

cPKCs and nPKCs are translocated from the soluble to the particulate fraction when activated by several stimuli (Kraft et al., 1982; Mochly-Rosen et al., 1990; Jaken, 1996; Ohno, 1997). The molecular mechanism of this translocation, however, has yet to be clarified. A system for monitoring the translocation of γ-PKC in living cells has recently been developed that uses protein fused with green fluorescent protein (GFP) (Sakai et al., 1997; Oancea et al., 1998). This experimental breakthrough enabled us to investigate the subspecies-specific function of PKC by analyzing the different targeting mechanisms of each subspecies.

Several saturated and unsaturated fatty acids also are reported to potentiate the activity of PKC (McPhail et al., 1984; Murakami et al., 1986; Shinomura et al., 1991; Asaoka et al., 1992; Nishizuka, 1995). For example, saturated fatty acids that have carbon chain lengths of C13 to C18 activate γ- and ε-PKC in vitro (Kasahara and Kikkawa, 1995), and unsaturated fatty acids, such as arachidonic and oleic acid, enhance the kinase activity of several PKC subspecies (Sekiguchi et al., 1988; Lester et al., 1991; Shinomura et al., 1991; Chen and Murakami, 1992). These fatty acid–induced activations of PKCs are enhanced by the presence of diacylglycerol. Furthermore, arachidonic acid–induced translocation of ε-PKC and oleic acid–induced translocation of α-, βII-, and δ-PKC have been shown by immunoblot analysis (Khan et al., 1993; Huang et al., 1997). Less information, however, is available on the underlying mechanism of the effects of fatty acids on PKC translocation. To clarify the physiological involvement of fatty acids in PKC signaling pathways, we studied the effects of 11 fatty acids on the translocation of γ- and ε-PKC in living cells that expressed PKC subspecies fused with GFP.

Materials and Methods

Materials

Tridecanoic acid, myristic acid, pentadecanoic acid, palmitic acid, heptadecanoic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, arachidonic acid, and docosahexaenoic acid were purchased from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ). 1,2-dioctanoylglycerol (DiC8) and BAPTA-AM were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA) and Research Biochemicals International (Natick, MA), respectively. All the other chemicals used were of analytical grade.

Cell Culture

COS-7 cells were purchased from the Riken cell bank (Tsukuba, Japan). The CHO-K1 cell strain was a gift from Dr. Nishijima (National Institute of Health, Tokyo, Japan). COS-7 cells were cultured in DME, and CHO-K1 cells in Ham's F12 medium (GIBCO BRL, Grand Island, NY) at 37°C in a humidified atmosphere containing 5% CO_2. Both media contained 25 mM glucose, and both were buffered with 44 mM $NaHCO_3$ and supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The FBS used was not heat inactivated.

Construct of Plasmids Encoding the γ- and ε-PKC–GFP Fusion Proteins

The plasmid bearing humanized GFP-S65A/Y145F cDNA was donated by Dr. Umesono (Kyoto University, Kyoto, Japan). A cDNA fragment encoding GFP with a HindIII site in the 5′-terminal end and an EcoRI site in the 3′-terminal end were obtained by a PCR with pCMX-SAH/Y145F as the template. The sense primer was 5′-AAGCTTATGGTGAGCAAGGGCGAGGAG-3′, and the antisense primer was 5′-TTGAATTCCTAGCTAGCTGGCCAGGATCC-3′.

Rat γ-PKC cDNA was obtained from the cDNA clone of λCKRγ1 (Ono et al., 1988*a*). After digestion with EcoRI, an insert fragment encoding rat γ-PKC was subcloned to an expression plasmid for mammalian cells, pTB 701 (Ono et al., 1988*b*) (designated BS 55). A cDNA fragment of γ-PKC with an EcoRI site in the 5′ terminus and a HindIII site in the 3′ terminus also was produced by a PCR with BS 55 as the template. The sense primer was 5′-TTGAATTCATGGCGGGTCTGGGTCCTGG-3′, and the antisense primer was 5′-TTAAGCTTATGGCGGGTCTGGGTCCTGG-3′. The PCR products of both GFP and γ-PKC were digested with EcoRI and HindIII and then subcloned together into the EcoRI site in pTB701 (BS 336).

A cDNA fragment encoding GFP with MunI-EcoRI-BglII sites in the 5′ terminus and a MunI site in the 3′ terminus was obtained by PCR with pCMX-SAH/Y145F as the template. The sense primer was 5′-TTTCAATTGAATTCAGATCTATGGTGAGCAAGGGCGAGGAG-3′, and the antisense primer was 5′-GGCAATTGCTAGCTAGCTGGCCAGGATCC-3′. The PCR product was subcloned into pTB 701 (BS 340). The plasmid bearing rat ε-PKC cDNA in pTB 701 was a gift from Dr. Ono (Kobe University) (BS 254). A cDNA fragment of ε-PKC with a BglII site in the 5′ and 3′ termini was produced by a PCR with BS 254 as the template. The sense primer was 5′-TTAGATCTACCATGGTAGTGTTCAATGG-3′, and the antisense primer was 5′-TTAGATCTGGGCATCAGGTCTTCACCAAA-3′. The PCR product for ε-PKC was digested with BglII and subcloned to the BglII site in BS 340 (BS 394).

Kinase Assay of Native ε-PKC and ε-PKC–GFP

COS-7 cells were transiently transfected by electroporation with plasmids encoding ε-PKC and ε-PKC–GFP and then cultured. ε-PKC and ε-PKC–GFP were immunoprecipitated with anti–ε-PKC monoclonal antibody (Transduction Laboratories, Lexington, KY), and their kinase activities were assayed as described elsewhere (Sakai et al., 1997). In brief, the immunoprecipitate was suspended in 120 μl of Dulbecco's PBS (−), and 10 μl of the suspended pellet was used for the kinase assay. Kinase activity measurements of the immunoprecipitated ε-PKC and ε-PKC–GFP were based on the incorporation of ^{32}P into a fragment of myelin basic protein (Sigma Chemical Co., St. Louis, MO) from [γ-^{32}P]ATP in the presence of 8 μg/ml phosphatidylserine (PS) and 0.8 μg/ml diolein (DO). Basal activity was measured in the presence of 0.5 mM EGTA instead of PS or DO.

Transfection of PKCs and Fusion Proteins to Cultured CHO-K1 Cells

Plasmids (~5.5 μg) encoding γ- or ε-PKC–GFP (BS 336 and BS 394) were transfected to 5×10^6 CHO-K1 cells by lipofection using TransIT™-LT2 (Mirus Co., Madison, WI) according to the manufacturer's standard protocol. The transfected cells were cultured at 37°C to obtain the optimal GFP fluorescence and then used for immunoblotting, immunostaining, and the observation of translocation.

Immunostaining of CHO-K1 Cells Expressing ε-PKC and Its Fusion Protein

Before and after translocation was induced by 1 μM TPA, cells expressing ε-PKC or ε-PKC–GFP were fixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M PBS for 30 min. After three washes with 0.1 M PBS, the cells were treated with PBS containing 0.3% Triton X and 10% normal goat serum (NGS) for 20 min. They then were incubated sequentially, first with anti–ε-PKC monoclonal antibody (diluted 1:1,000) for 40 min in PBS containing 0.03% Triton X (PBS-T) and then with Cy3-labeled goat anti–mouse IgG (Amersham Corp., Arlington Heights, IL) for 30 min at room temperature. Fluorescence of ε-PKC–like immunoreactivity was observed under a confocal laser scanning fluorescent microscope (model LSM 410 invert; Carl Zeiss, Jena, Germany) at 588-nm argon excitation with a 590-nm-long pass barrier filter. GFP fluorescence was observed at 488-nm argon excitation with a 510–525-nm-band pass barrier filter.

Observation of γ- and ε-PKC–GFP Translocation

CHO-K1 cells expressing γ- and ε-PKC–GFP were spread on glass-bottomed culture dishes (MatTek Corp., Ashland, MA) and incubated for 16–60 h before observation. The culture medium was replaced with normal Hepes buffer composed of 135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$,

1.8 mM $CaCl_2$, 5 mM Hepes, and 10 mM glucose, pH 7.3. In the experiment with Ca^{2+} chelators, the cells were washed with Hepes buffer without $CaCl_2$ (Ca^{2+}-free Hepes buffer) and then incubated for 30 min with 2.5 mM EGTA and 30 μM BAPTA-AM in Ca^{2+}-free Hepes buffer. Translocation of the fusion protein was triggered by the addition of the various stimulants to the Hepes buffer to obtain the appropriate final concentration. All experiments were done at 37°C.

The fluorescence of GFP was monitored under a confocal laser scanning fluorescent microscope at 488-nm argon excitation with a 515-nm-long pass barrier filter. Lastly, the changes in fluorescence were quantified using confocal software.

Codetection of the Golgi Network and ε-PKC–GFP Translocated by the Stimulation of Arachidonic Acid

Texas red–conjugated wheat germ agglutinin was used to monitor the Golgi network. After induction of the translocation of ε-PKC–GFP by 100 μM arachidonic acid, the cells were fixed and treated with 0.3% Triton X and 10% NGS as described above, after which the cells were incubated with 0.5 μg/ml Texas red–conjugated wheat germ agglutinin (Molecular Probes, Leiden, Netherlands) in PBS-T for 30 min. Finally, the fluorescence of Texas red and GFP were observed under a confocal laser scanning fluorescent microscope, the former at 588-nm argon excitation using a 590-nm-long pass barrier filter and the latter at 488-nm argon excitation using a 510–525-nm-band pass barrier filter.

Results

Properties of ε-PKC and Its Fusion Protein with GFP

Enzymological and immunochemical properties of ε-PKC–GFP and native ε-PKC were examined. Fig. 1 *A* shows that both the immunoprecipitated ε-PKC and ε-PKC–GFP had basal kinase activity and that both were activated at ~3.3-fold the basal level in the presence of PS and DO. These findings indicate that ε-PKC–GFP also is dependent

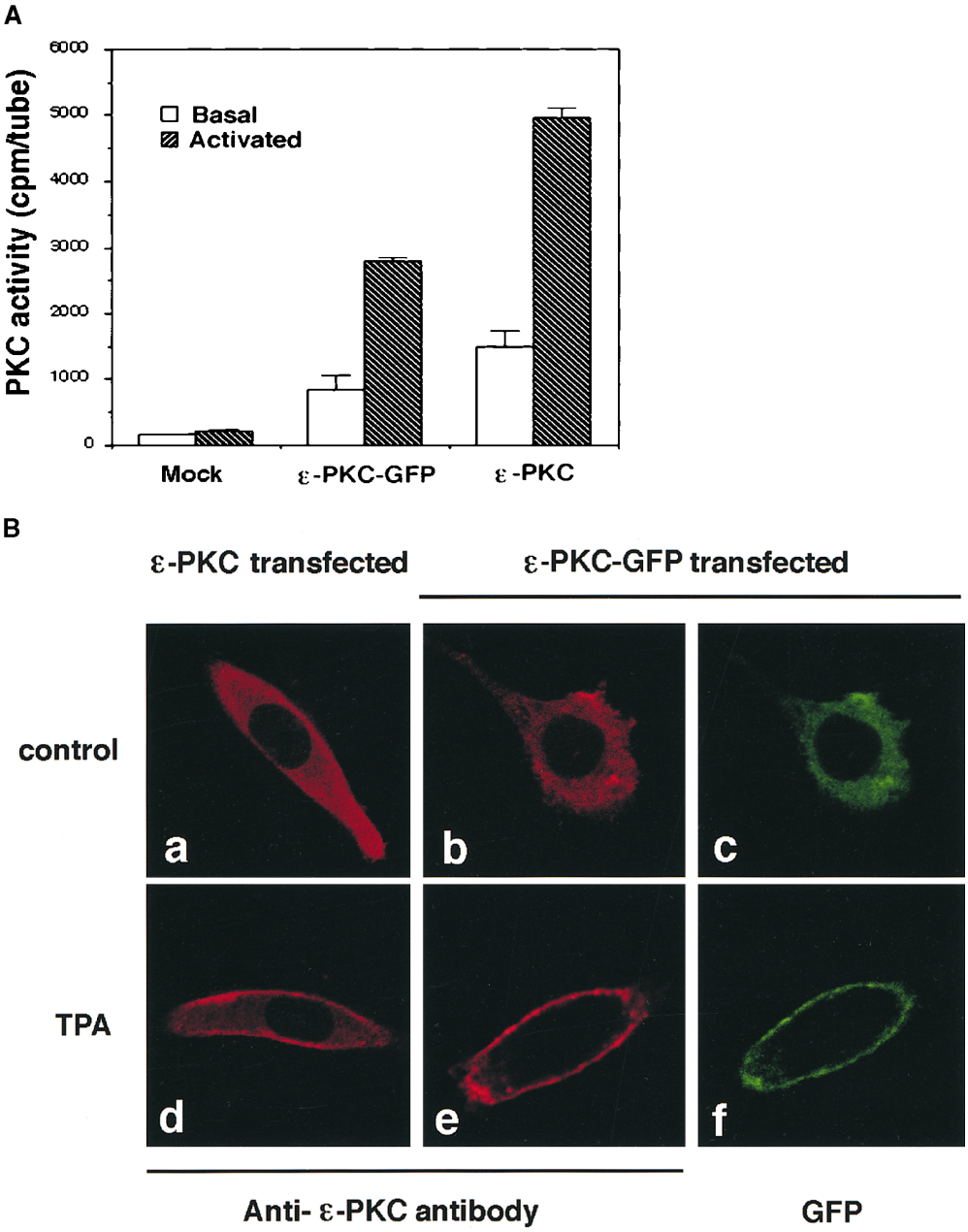

Figure 1. Comparison of properties of native ε-PKC and its fusion protein with those of GFP. (*A*) Kinase activities of immunoprecipitated ε-PKC and ε-PKC–GFP were measured in the presence (activated; *hatched bar*) or absence of (basal; *open bar*) PS and DO. (*B*) Fluorescent microscopic photos of CHO-K1 cells expressing ε-PKC or ε-PKC–GFP. Transfected CHO-K1 cells were fixed before or after treatment with 1 μM TPA. Immunoreactivity of ε-PKC (*a* and *d*) or ε-PKC–GFP (*b* and *e*) was made visible with anti–ε-PKC antibody (*red*). The fluorescence of ε-PKC–GFP (*c* and *f*) was simultaneously observed with a confocal laser scanning fluorescent microscope (*green*), as described in Materials and Methods. ε-PKC immunoreactivity was translocated from the cytoplasm to the plasma membrane by TPA treatment, both in ε-PKC (*a* and *d*) and ε-PKC–GFP (*b* and *e*) expressing CHO-K1 cells. The localizations of ε-PKC immunoreactivity and GFP fluorescence were indistinguishable, as seen in *b* and *c*. Moreover, localization was indistinguishable after TPA treatment (*e* and *f*).

on PS and DO and that its enzymological property is very similar to that of the native ε-PKC.

Immunostaining with anti–ε-PKC antibodies showed that both ε-PKC and ε-PKC–GFP were expressed in the cytoplasm of CHO-K1 cells before stimulation (Fig. 1 *B*, *a* and *b*) and intense immunoreactivities on the plasma membrane after treatment with 1 μM TPA (Fig. 1 *B*, *d* and *e*). Within the same cells, localization of the GFP fluorescence of ε-PKC–GFP (Fig. 1 *B*, *b*) corresponded to that of the ε-PKC–like immunoreactivity (Fig. 1 *B*, *c*). Colocalization of GFP and ε-PKC–like immunoreactivity also was observed after treatment with TPA (Fig. 1 *B*, *e* and *f*). These results indicate that in response to TPA, both native ε-PKC and ε-PKC–GFP were translocated from the cytoplasm to the plasma membrane without proteolysis. This was confirmed by immunoblotting using anti–ε-PKC antibody (data not shown).

Effects of Fatty Acids on the Translocation of γ- and ε-PKC–GFP

The effect of a single application of a saturated fatty acid (such as tridecanoic acid, myristic acid, pentadecanoic acid, palmitic acid, heptadecanoic acid, or stearic acid) on the subcellular localization of γ-PKC–GFP was investigated. All the saturated fatty acids examined induced the translocation of γ-PKC–GFP at 200 μM. Fig. 2 shows sequential pictures of the typical translocation of γ-PKC–GFP induced by saturated fatty acids. When γ-PKC–GFP was expressed in CHO-K1 cells, intense fluorescence was present throughout the cytoplasm, and faint fluorescence was present in the nucleus. The presence of γ-PKC–GFP in the nucleus was obvious because the nucleolus appeared as a small black circle. Stimulation with 200 μM tridecanoic acid, pentadecanoic acid, or palmitic acid caused translocation of the γ-PKC–GFP in the cytoplasm to the plasma membrane at 20 s, but there was a return to the cytoplasm within 2 min. These fatty acids, however, had no effect on the γ-PKC–GFP in the nucleus. These fluorescence changes were confirmed by the profiles of GFP intensity shown in Fig. 2. Myristic acid, heptadecanoic acid, and stearic acid also induced translocation of γ-PKC–GFP similar to that shown in Fig. 2 (data not shown).

Unsaturated fatty acids also caused the translocation of γ-PKC–GFP. Oleic and arachidonic acid at 200 μM induced a very rapid, transient translocation of γ-PKC–GFP from the cytoplasm to the plasma membrane similar to that of saturated fatty acids (Fig. 3). Linolenic and docosahexaenoic acids induced γ-PKC–GFP translocation simi-

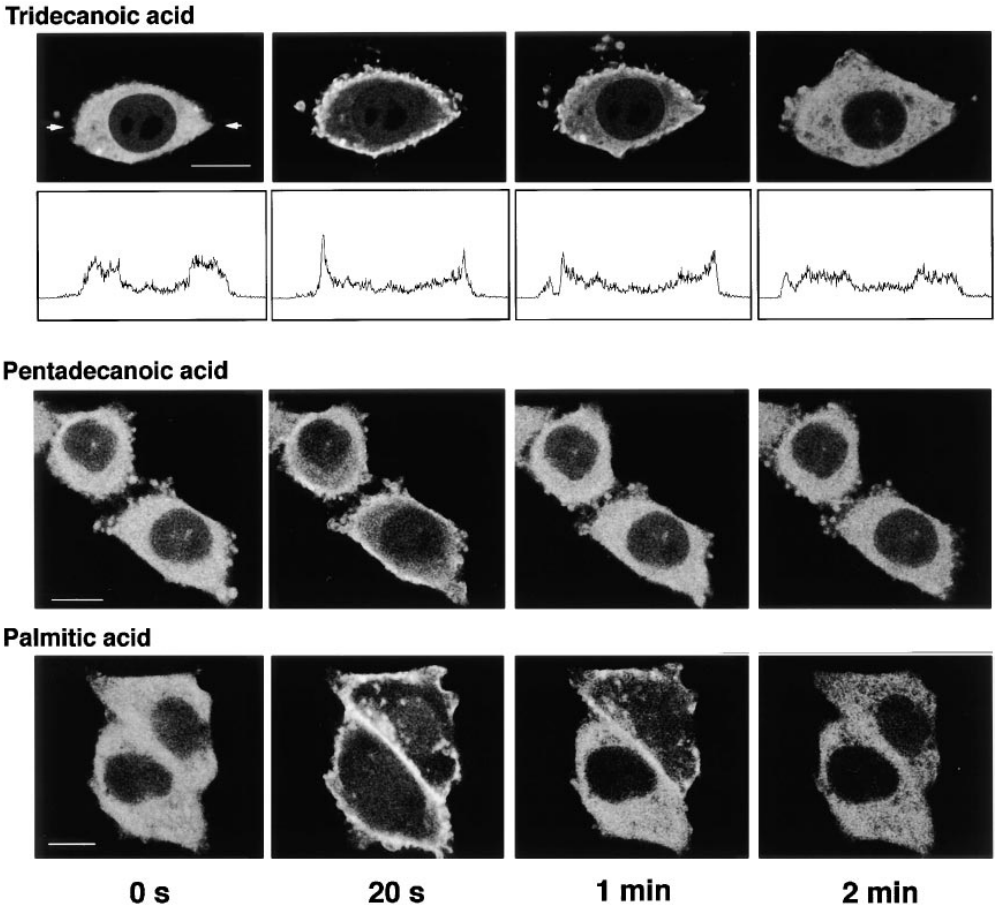

Figure 2. Translocation of γ-PKC–GFP induced by saturated fatty acids in CHO-K1 cells. (*Top row*) Changes induced by 200 μM tridecanoic acid in the fluorescence of γ-PKC–GFP expressed in CHO-K1 cells. γ-PKC–GFP fusion protein is present throughout the cytoplasm within the transfected CHO-K1 cells, and faint fluorescence is seen in the nucleus. The addition of 200 μM tridecanoic acid induced rapid translocation of γ-PKC–GFP fluorescence from the cytoplasm to the plasma membrane, which took place within 20 s of stimulation. Thereafter, γ-PKC–GFP quickly was retranslocated from the membrane to cytoplasm within 2 min, reaching a state similar to that before stimulation. (*Second row*) Changes in the profiles of GFP intensity on the same axis across a cell treated with 200 μM tridecanoic acid. The axis is between the arrows in the upper left photo. (*Third row*) Changes produced by 200 μM pentadecanoic acid in the fluorescence of γ-PKC–GFP expressed in CHO-K1 cells. The translocation of γ-PKC–GFP induced by pentadecanoic acid is similar to that of tridecanoic acid. (*Bottom row*) Changes induced by 200 μM palmitic acid in the fluorescence of γ-PKC–GFP expressed in CHO-K1 cells. The translocation is similar to the translocations of tridecanoic and pentadecanoic acids. Bars, 10 μm.

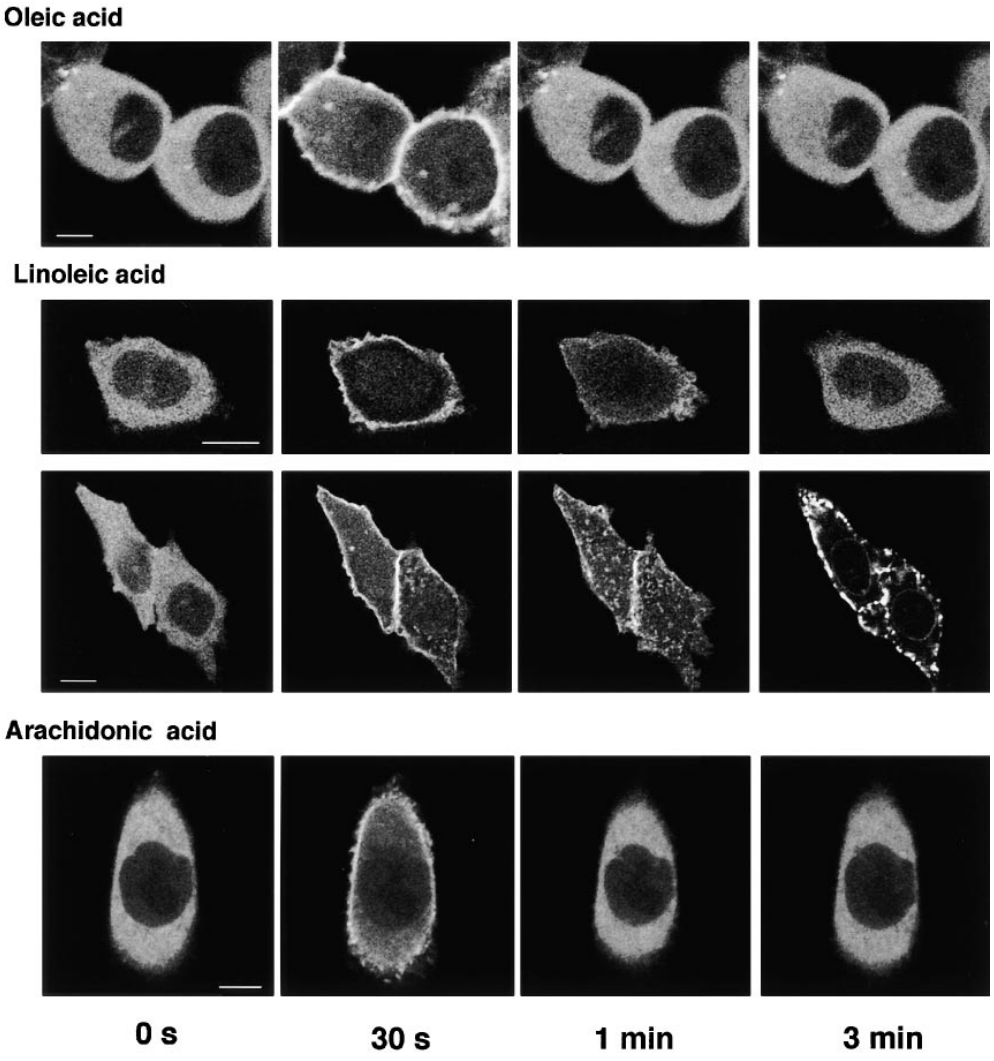

Figure 3. Translocation of γ-PKC–GFP induced by unsaturated fatty acids. (*Top row*) Changes induced by 200 μM oleic acid in the fluorescence of γ-PKC–GFP. Oleic acid also induced very rapid, transient translocation of γ-PKC–GFP from the cytoplasm to the plasma membrane that was similar to that induced by saturated fatty acid. (*Second and third rows*) Changes induced by 100 and 200 μM linoleic acid in the fluorescence of γ-PKC–GFP. Linoleic acid at a low concentration (100 μM) induced translocation of γ-PKC–GFP from the cytoplasm to the plasma membrane as did oleic acid. At 200 μM, however, it caused a different translocation of γ-PKC–GFP. After rapid translocation to the membrane at 30 s, the γ-PKC–GFP on the plasma membrane faded slightly. γ-PKC–GFP is seen as dots throughout the cytoplasm at 1 min, after which it appears on the plasma membrane as patchy dots and on the nuclear membrane. (*Bottom row*) Changes induced by 200 μM arachidonic acid in the fluorescence of γ-PKC–GFP. The translocation of γ-PKC–GFP induced by arachidonic acid is similar to that induced by saturated fatty acid and oleic acid. Bars, 10 μm.

lar to that of oleic and arachidonic acids (data not shown). Linoleic acid at 200 μM, however, occasionally translocated γ-PKC–GFP to different components, but at a lower concentration (100 μM), it induced a translocation from the cytoplasm to the membrane similar to that of oleic and arachidonic acids (Fig. 3). After rapid translocation to the membrane caused by treatment with 200 μM linoleic acid, the γ-PKC–GFP on the plasma membrane faded slightly, and at 1 min it appeared as an accumulation of dots throughout the cytoplasm. Finally, γ-PKC–GFP again appeared as an accumulation of patchy dots on the plasma membrane and on the nuclear membrane. It is noteworthy that the target site for γ-PKC–GFP on stimulation with linoleic acid depends on the concentration.

Translocation of γ-PKC–GFP was also examined in response to various concentrations of the fatty acids (Table I). Of the 11 fatty acids tested, γ-PKC–GFP was most sensitive to pentadecanoic acid; ~38% of the cells showed marked translocation in response to 50 μM pentadecanoic acid. Tridecanoic acid, palmitic acid, heptadecanoic acid, linoleic acid, and docosahexaenoic acid at 100 μM caused translocation in more than 40% of the cells, whereas the other fatty acids required more than 200 μM.

The sensitivity of ε-PKC–GFP to fatty acids differed from that of γ-PKC–GFP (Table I). Except for tridecanoic, linoleic, and arachidonic acids, the fatty acids in general had little effect on the translocation of ε-PKC–GFP. As shown in Table I, only five fatty acids (tridecanoic, pentadecanoic, linoleic, arachidonic, and docosahexaenoic acids) induced translocation when used alone at 200 μM. The other fatty acids required more than 300 μM for activity or failed to translocate ε-PKC–GFP. Tridecanoic acid was the most effective for the ε-PKC–GFP translocation, being more effective for ε-PKC–GFP than γ-PKC–GFP (Table I).

ε-PKC–GFP fluorescence was detected in the cytoplasm. Occasionally, fairly intense fluorescence was present in the perinuclear region (Fig. 4 *A, second row*), but no ε-PKC–GFP was present within the nucleus before stimulation. Except for palmitic acid, the saturated fatty acids

Table I. Effectiveness of Fatty Acids in Inducing the Translocation of γ- and ε-PKC–GFP

	γ-PKC–GFP					ε-PKC–GFP				
Fatty acid	25	50	100	200	300	25	50	100	200	300
			μM					*μM*		
Tridecanoic acid	0 ($n = 8$)	0 ($n = 4$)	47.1 ($n = 17$)	71.4 ($n = 7$)	100 ($n = 4$)	0 ($n = 6$)	46.2 ($n = 9$)	64.3 ($n = 14$)	75.0 ($n = 16$)	NT
Myristic acid	NT	NT	0 ($n = 6$)	50.0 ($n = 6$)	83.3 ($n = 6$)	NT	NT	0 ($n = 6$)	0 ($n = 4$)	66.6 ($n = 9$)
Pentadecanoic acid	NT	37.5 ($n = 8$)	82.3 ($n = 18$)	100 ($n = 5$)	NT	NT	NT	0 ($n = 11$)	68.8 ($n = 16$)	100 ($n = 4$)
Palmitic acid	NT	0 ($n = 4$)	46.0 ($n = 13$)	71.4 ($n = 7$)	78.0 ($n = 9$)	NT	NT	0 ($n = 5$)	0 ($n = 4$)	0 ($n = 5$)
Heptadecanoic acid	NT	0 ($n = 12$)	62.5 ($n = 8$)	100 ($n = 4$)	NT	NT	NT	0 ($n = 5$)	0 ($n = 4$)	40.0 ($n = 5$)
Stearic acid	NT	0 ($n = 4$)	38.4 ($n = 13$)	62.5 ($n = 8$)	83.3 ($n = 6$)	NT	0 ($n = 4$)	0 ($n = 7$)	12.5 ($n = 8$)	85.7 ($n = 7$)
Oleic acid	NT	NT	14.3 ($n = 18$)	63.0 ($n = 11$)	77.8 ($n = 9$)	NT	NT	0 ($n = 5$)	0 ($n = 6$)	20.0 ($n = 10$)
Linoleic acid*	NT	0 ($n = 4$)	42.1 ($n = 19$)	76.5 ($n = 17$)	87.5 ($n = 8$)	NT	28.9 ($n = 7$)	42.8 ($n = 4$)	61.5 ($n = 13$)	100 ($n = 4$)
Linolenic acid	NT	NT	20.0 ($n = 15$)	55.5 ($n = 9$)	75.5 ($n = 4$)	NT	NT	0 ($n = 4$)	0 ($n = 5$)	20.0 ($n = 5$)
Arachidonic acid*	NT	NT	0 ($n = 13$)	50 ($n = 18$)	75 ($n = 4$)	NT	25.0 ($n = 4$)	80.0 ($n = 20$)	80.0 ($n = 5$)	88.9 ($n = 9$)
Docosahexaenoic acid	NT	0 ($n = 4$)	41.1 ($n = 17$)	66.6 ($n = 11$)	100 ($n = 5$)	0 ($n = 4$)	0 ($n = 4$)	30.7 ($n = 13$)	100 ($n = 4$)	NT

*Linoleic acid and arachidonic acid induced translocation of ε-PKC from the cytoplasm to the perinuclear area, which occurred somewhat slowly. A high concentration of linoleic acid sometimes translocated γ-PKC in the nucleus to the nuclear membrane. The other fatty acids tested caused rapid, transient translocation of γ- and ε-PKC from the cytoplasm to the plasma membrane. Numbers show the percentages of cells that showed marked translocation in response to each concentration of the fatty acid. *n*, number of cells used in the experiment; *NT*, not tested.

induced rapid, transient translocation of ε-PKC–GFP from the cytoplasm to the membrane (Fig. 4 *A*). An addition of 200 μM tridecanoic and pentadecanoic acid caused rapid movement of ε-PKC–GFP to the membrane and a return to the cytoplasm at 2 min. Of the five unsaturated fatty acids tested, the translocation of ε-PKC–GFP was reproducibly induced by linoleic, arachidonic, or docosahexaenoic acid at 200 μM. The translocation induced by docosahexaenoic acid was similar to that induced by the saturated fatty acids in Fig. 4 *A* (data not shown). Linoleic and arachidonic acids, however, induced a unique translocation of ε-PKC–GFP (Fig. 4 *B*). Neither linoleic nor arachidonic acid at 200 μM induced rapid, reversible translocation of ε-PKC–GFP to the plasma membrane, but both induced slow, irreversible accumulation of ε-PKC–GFP to perinuclear regions. The accumulation of ε-PKC–GFP in the perinuclear region was evident 3 min after the stimulations with linoleic and arachidonic acids and was still detectable 15 min after stimulation (data not shown). Arachidonic acid–induced translocation occurred in the presence of 10 μM of indomethacine, which inhibits the generation of eicosanoids from arachidonic acid (data not shown) (Flower et al., 1985).

Immunostaining with anti–γ-PKC and –ε-PKC antibodies confirmed that these fatty acid–induced translocations occurred in CHO-K1 cells that expressed native γ- and ε-PKC (data not shown).

Target Site of ε-PKC–GFP on Stimulation with Arachidonic Acid

To identify the compartment in which ε-PKC–GFP accumulated in response to arachidonic acid, the Golgi network was made visible with Texas red–conjugated wheat germ agglutinin after arachidonic acid induced the translocation of ε-PKC–GFP.

GFP fluorescence was present throughout the cytoplasm, but not in the nucleus, and was most intense in the perinuclear region (Fig. 5, *left*). The fluorescence of the two Texas red–stained cells shown in a micrograph (Fig. 5, *center*) indicates that one was the cell detected by GFP fluorescence and the other was a cell that expressed no ε-PKC–GFP. Intense Texas red fluorescence was present around the nucleus. An overlapping image shows that the Texas red fluorescence and GFP colocalized in the perinuclear region (Fig. 5, *right*).

Effect of Ca^{2+} Chelators on Fatty Acid–induced Translocation of γ- and ε-PKC–GFP

To clarify whether the fatty acid–induced translocations of γ- and ε-PKC–GFP require Ca^{2+} ions, we examined the effects of Ca^{2+} chelators on the translocations. Pretreatment with 2.5 mM EGTA and 30 μM BAPTA-AM blocked the translocation of γ-PKC–GFP induced by docosahexaenoic acid, but not that of ε-PKC–GFP (Fig. 6). Similarly, tridecanoic acid–induced translocation of γ-PKC was inhibited by Ca^{2+} chelators, whereas translocation of ε-PKC occurred in the presence of Ca^{2+} chelators (data not shown). These findings suggest that the fatty acid–induced translocation of ε-PKC–GFP is independent of Ca^{2+}, whereas Ca^{2+} is necessary for the fatty acid–induced translocation of γ-PKC.

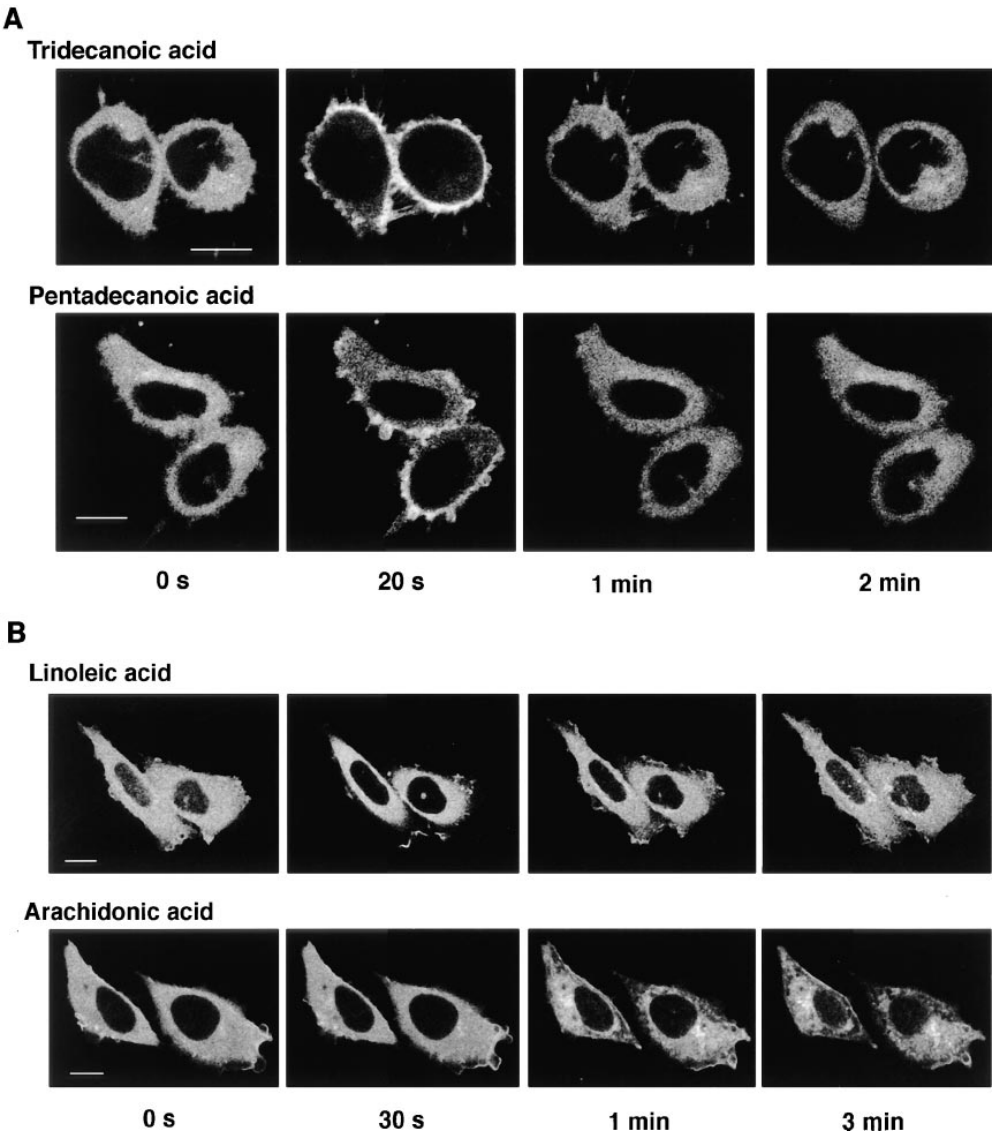

Figure 4. Translocation of ϵ-PKC–GFP induced by saturated and unsaturated fatty acids. (*A*) Translocation of ϵ-PKC–GFP induced by saturated fatty acids. (*Top row*) Changes induced by 200 μM tridecanoic acid in the fluorescence of ϵ-PKC–GFP expressed in CHO-K1 cells. ϵ-PKC–GFP fusion protein is present throughout the cytoplasm but not in the nucleus. The addition of 200 μM tridecanoic acid induced rapid translocation of ϵ-PKC–GFP fluorescence from the cytoplasm to the plasma membrane, within 20 s of stimulation. Thereafter, ϵ-PKC–GFP was rapidly retranslocated from the membrane to the cytoplasm. (*Second row*) Changes induced by 200 μM pentadecanoic acid in the fluorescence of ϵ-PKC–GFP. Fairly intense fluorescence is present in the perinuclear region before the stimulation. The translocation of ϵ-PKC–GFP induced by pentadecanoic acid is similar to that of tridecanoic acid. (*B*) Translocation of ϵ-PKC–GFP induced by unsaturated fatty acids. (*Third row*) Changes induced by 200 μM linoleic acid in the fluorescence of ϵ-PKC–GFP. The addition of 200 μM linoleic acid induced slow translocation of ϵ-PKC–GFP fluorescence from the cytoplasm to the perinuclear region. Intense dotlike fluorescence is present near the nucleus at 3 min. (*Bottom row*) Changes induced by 200 μM arachidonic acid in the fluorescence of ϵ-PKC–GFP. ϵ-PKC–GFP fluorescence in the cytoplasm has faded, and intense fluorescence is present in the perinuclear area at 1 min. The accumulation of ϵ-PKC–GFP at the perinuclear area is still detectable at 3 min. Bars, 10 μm.

Synergistic Effect of Arachidonic Acid and Diacylglycerol on the Translocation of γ- and ϵ-PKC–GFP

As shown in Fig. 7, DiC8 at 10 μM caused very rapid translocation of γ-PKC–GFP to the plasma membrane at 15 s, but the translocated γ-PKC–GFP returned to the initial state within 3 min (Fig. 7 *A*), and thereafter no translocation occurred. The application of 1 μM DiC8 alone, however, failed to induce the γ-PKC–GFP translocation (Fig. 7 *B*). Similarly, use of arachidonic acid alone at 200 μM, but not at 25 μM, caused translocation of γ-PKC–GFP (Table I). Simultaneous applications of 1 μM DiC8 and 25 μM arachidonic acid induced remarkable translocation of γ-PKC–GFP. 15 s after the coapplication, there was very faint translocation to the plasma membrane, similar to that induced by a single application of a low concentration of DiC8 or arachidonic acid. Subsequently, about 3 min after stimulation, slow translocation of γ-PKC–GFP to the plasma membrane became evident. This late phase translocation was unidirected and irreversible (Fig. 7 *C*). This synergistic action occurred in the presence of 10 μM indomethacine, indicating that arachidonic acid itself contributed to the unidirected and irreversible translocation of γ-PKC–GFP (data not shown). In contrast, the translocation of ϵ-PKC–GFP was neither markedly enhanced nor altered by the simultaneous application of arachidonic acid and DiC8.

Discussion

Using γ- and ϵ-PKC proteins fused with GFP, we showed

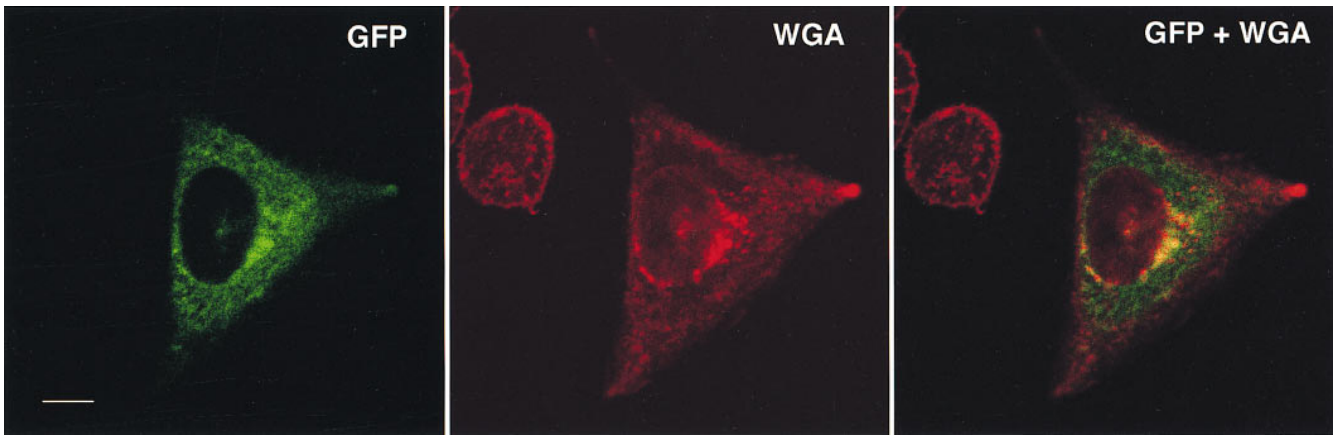

Figure 5. Colocalization of ε-PKC–GFP and wheat germ agglutinin binding sites in ε-PKC–GFP–expressing CHO-K1 cells treated with arachidonic acid. CHO-K1 cells transfected with ε-PKC–GFP were fixed after treatment with 100 μM arachidonic acid. Cells were treated with Texas red–conjugated wheat germ agglutinin to make the Golgi network visible. The localization of ε-PKC–GFP is shown (at left in *green*) by making GFP visible. The Golgi network is shown in the center (*red*). The overlapping images of GFP and Texas red appear as yellow. Bar, 10 μm.

the fatty acid–induced translocation of PKCs in living cells in real time. In order for the proteins' movement to be visible by monitoring the fluorescence of GFP fused to the protein, the fusion proteins must have the same properties as native PKCs. Furthermore, because PKCs are reported to be proteolysed by proteases such as calpain (Kishimoto et al., 1983), we examined the enzymological and immunochemical properties of γ- and ε-PKC–GFP. Because γ-PKC–GFP is reported to have the same properties as native γ-PKC (Sakai et al., 1997), we analyzed the properties of ε-PKC–GFP by a kinase assay, immunostaining, and Western blotting and found that (*a*) the activity of ε-PKC–GFP was dependent on PS and DO (Fig. 1 *A*); (*b*) the ε-PKC–GFP images immunostained with anti–ε-PKC antibody coincided with the fluorescences of GFP before and after TPA stimulation (Fig. 1 *B*); and (*c*) no ε-PKC–GFP–degraded product was found in the immunoblot analysis, even after TPA treatment (data not shown). These findings indicate that the GFP fluorescence fused to ε-PKC can be used as a marker for native ε-PKC.

We investigated the effects of saturated fatty acids with carbon chain lengths of C13 to C18 and unsaturated fatty acids on the translocation of γ- and ε-PKC–GFP because these fatty acids are known to potentiate the activity of PKC subspecies (Kasahara and Kikkawa, 1995). Of the 11 fatty acids examined, the translocation of γ-PKC was induced more effectively by the saturated than the unsaturated fatty acids, whereas the kinase activity was enhanced more effectively by the unsaturated fatty acids (Shinomura et al., 1991). Saturated fatty acids enhanced the kinase activity of both γ- and ε-PKC by the synergistic action of diacylglycerol, and ε-PKC was more sensitive to

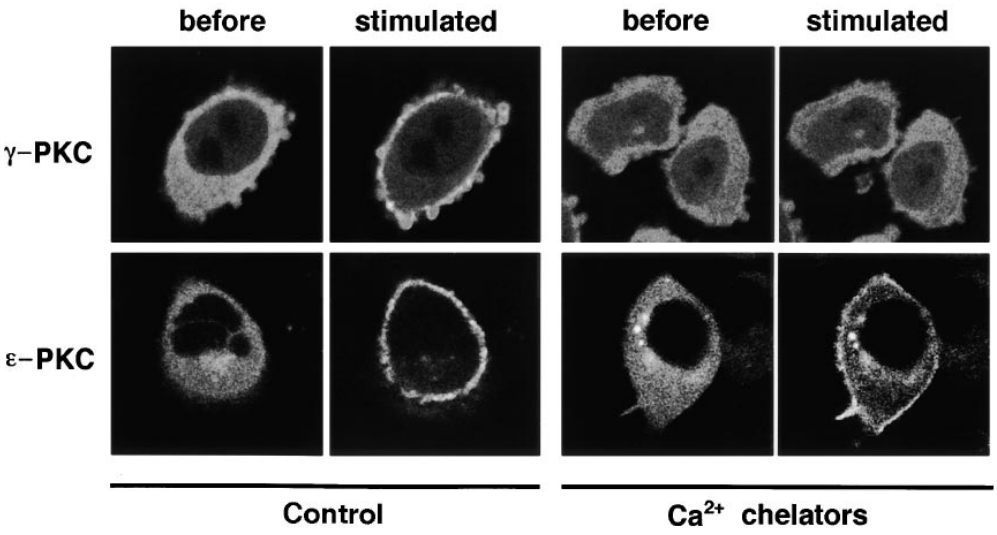

Figure 6. Effects of Ca^{2+} chelators on fatty acid–induced translocation of γ- and ε-PKC–GFP. (*Control*) CHO-K1 cells expressing γ- and ε-PKC–GFP were incubated in normal Hepes buffer for 30 min, and then docosahexaenoic acid was added to the buffer to give 200 μM. Images were recorded before treatment and at 30 s after treatment (*stimulated*). Docosahexaenoic acid–induced translocation occurred for both γ- and ε-PKC–GFP. (*Ca^{2+} chelators*) After the cells had been incubated for 30 min with 2.5 mM EGTA and 30 μM BAPTA-AM in Ca^{2+}-free Hepes buffer, docosahexaenoic acid was challenged as in the control. Images were recorded before treatment and 30 s after treatment (*stimulated*). Treatment with Ca^{2+} chelators blocked the docosahexaenoic acid–induced translocation of γ-PKC–GFP but not that of ε-PKC–GFP.

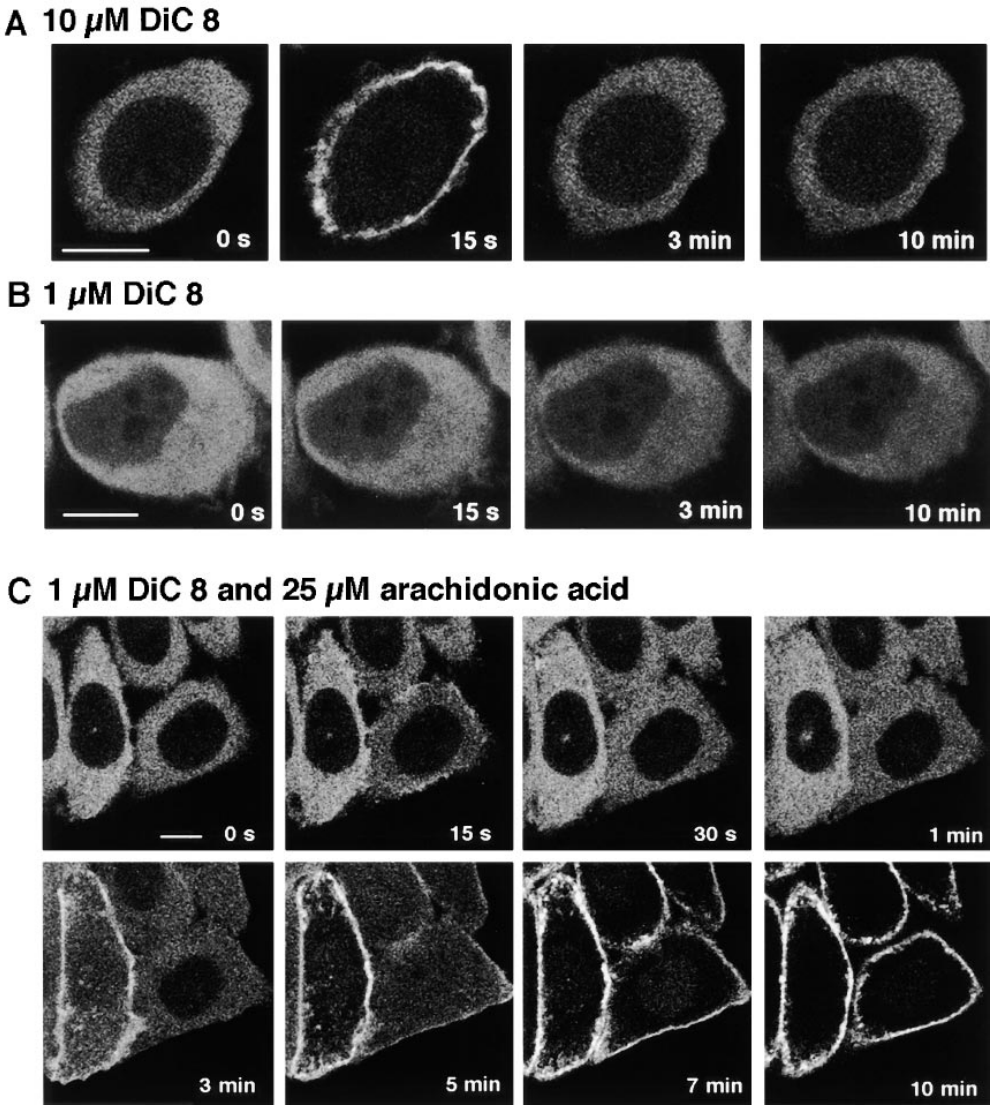

Figure 7. Synergistic effect of arachidonic acid and the diacylglycerol analogue on the translocation of γ-PKC–GFP. (*A*) The addition of 10 μM DiC8, a diacylglycerol analogue, induced rapid translocation of γ-PKC–GFP from the cytoplasm to the plasma membrane. (*B*) DiC8 at 1 μM did not translocate γ-PKC–GFP. (*C*) A coaddition of 25 μM arachidonic acid and 1 μM DiC8 induced rapid translocation of γ-PKC–GFP followed by delayed, irreversible translocation. A low concentration of DiC8 induced rapid, reversible translocation within 15 s when applied with a low concentration of arachidonic acid. After the rapid translocation, a second translocation occurred at 3 min, and γ-PKC–GFP remained on the membrane even 10 min after treatment. Bars, 10 μm.

saturated fatty acids than γ-PKC (Shinomura et al., 1991; Kasahara and Kikkawa, 1995). The translocation of ε-PKC, however, was less sensitive to the saturated fatty acids than was that of γ-PKC and was not induced synergistically in the presence of arachidonic acid and DiC8. These discrepancies may be due to the different methods used to detect the effects of the fatty acids; the translocation of PKC was monitored after the extracellular fatty acid application, whereas PKC activity was assayed by applying the fatty acids directly to the PKCs in vitro. Taking into account that translocation was induced by phorbol ester, even in the presence of a PKC inhibitor (Sakai et al., 1997), an increase in the kinase activity of PKC does not always correspond to the translocation of PKC.

Except for linoleic acid at a high concentration, all the fatty acids induced similar translocations of γ-PKC: rapid, reversible translocation from the cytoplasm to the plasma membrane. In contrast, both arachidonic and linoleic acids generated the slow translocation of ε-PKC from the cytoplasm to the perinuclear area, and saturated fatty acids induced a translocation of ε-PKC similar to that of γ-PKC. The target site of the arachidonic acid–induced translocation of ε-PKC was identified as the Golgi network. This is consistent with previous reports showing that ε-PKC was localized to the Golgi via its zinc-finger domain (Lehel et al., 1995) and that arachidonic acid stimulated ε-PKC redistribution in heart cells (Huang et al., 1997). These findings indicate that the target site of ε-PKC differs in response to the fatty acid used and strongly suggest that the mechanism of translocation induced by arachidonic acid differs for γ- and ε-PKC. These interpretations are supported by the different effects of fatty acids on the kinase activities of PKC subspecies in vitro (Shinomura et al., 1991; Koide et al., 1992; Kasahara and Kikkawa, 1995). Saturated fatty acids with carbon chain lengths of C12 to C14 activated α-, β-, γ-, and ε-PKC, but not δ-PKC. Phorbol ester synergically enhanced the activity of α-, β-, and γ- PKCs when simultaneously treated with fatty acids, whereas it suppressed the activity of ε-PKC.

Some fatty acids mobilize intracellular Ca^{2+} (Gamberucci et al., 1997; Schaloske et al., 1998). We therefore clarified whether fatty acid translocation depends on an

increase in the intracellular Ca^{2+} concentration. Docosahexaenoic and tridecanoic acids induced translocation of ϵ-PKC in the presence of Ca^{2+} chelators, indicating that the fatty acid–induced translocation of ϵ-PKC is independent of the increase in the intracellular Ca^{2+} concentration, although none of the fatty acids were tested in the presence of Ca^{2+} chelators. In contrast, neither docosahexaenoic nor tridecanoic acid caused the translocation of γ-PKC in the presence of Ca^{2+} chelators. This suggests that fatty acids cause the translocation of γ-PKC through an increase in the intracellular Ca^{2+} concentration, but it does not exclude the possibility that they directly act on γ- and ϵ-PKC and induce their translocation. Intracellular Ca^{2+} appears to be indispensable for γ-PKC translocation because the DiC8-induced translocation of γ-PKC was blocked by Ca^{2+} chelators (data not shown).

Furthermore, arachidonic acid increases the activities of γ-PKC and ϵ-PKC by the synergistic action of DiC8 (Kasahara and Kikkawa, 1995). As shown in Fig. 7, arachidonic acid also increased translocation sensitivity to DiC8 and then induced the additional, unidirectional translocation of γ-PKC. In contrast, synergistic translocation of ϵ-PKC could not be induced by arachidonic acid and DiC8. Oancea et al. (1998) also detected no synergistic effect of arachidonic acid on DiC8-induced translocation of the first cystein-rich region (Cys-1) of γ-PKC, but they did show that arachidonic acid inhibited the DiC8-induced translocation of the Cys-1 of PKC to the plasma membrane. These findings suggest that the synergistic effect of arachidonic acid on γ-PKC activity occurs through an unknown mechanism that differs from the targeting mechanism by arachidonic acid of Cys-1 (or ϵ-PKC) to the Golgi complex.

The anchoring proteins of PKC, RACKs, have recently been identified (Mochly-Rosen et al., 1991). RACKs bind activated PKC (Jaken, 1997) and are thought to be involved in PKC translocation. To clarify the mechanism of PKC translocation, it is necessary to examine the effects of these anchoring proteins on PKC translocation using GFP-labeled PKC in living cells.

In conclusion, each PKC subspecies has a different targeting mechanism that depends on the extracellular stimuli used, i.e., the fatty acid. Moreover, the synergistic actions of intracellular activators have an important role in targeting, thereby contributing to the subspecies-specific function.

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