Biochemical Pharmacology, Vol. 53, pp. 1683–1694, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00088-9

Receptor-mediated Diacylglycerol Kinase Translocation Dependent on Both Transient Increase in the Intracellular Calcium Concentration and Modification by Protein Kinase C

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ABSTRACT. Diacylglycerol kinase (DG kinase) is activated by various stimuli in many types of cells. We reported earlier that carbachol (CCh) induced DG kinase translocation from the cytosolic fraction to the membrane fraction in guinea pig taenia coli (*Biochem. Pharmacol.*, **50:** 591–599, 1995). In this study, the regulation mechanisms of DG kinase translocation are reported, based on the following findings: 1) CCh sustained an increase in DG kinase in the membrane fraction and a decrease in the cytosolic fraction; 2) blocking calcium influx by removing extracellular calcium did not affect the CCh-induced sustained DG kinase translocation; 3) exposing purified protein kinase C (PKC) to DG kinase increased DG kinase affinity to octylglycoside micelles only with the enzyme extracted from the cytosolic fraction; and 4) CCh-induced DG kinase translocation was reversed by removing CCh, and the serine/threonine phosphatase inhibitor, okadaic acid, blocked the reversal of the translocation. These results suggest that CCh-induced DG kinase translocation is promoted by both a transient increase in intracellular calcium, which may be released from the intracellular store, and by DG kinase phosphorylation by PKC. BIOCHEM PHARMACOL 53;11:1683–1694, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. 1-stearoyl-2-arachidonyl-*sn*-glycerol; dioctanoyl-*sn*-glycerol; okadaic acid; phosphatidic acid; signal transduction

DG is a representative second messenger which is generated immediately after receptor stimulation in many types of cells [1]. DG has been well studied as an endogenous activator of PKC [2–4], as has its relationship to phospholipase C as a principal formation route [5, 6]. DG kinase phosphorylates DG and is partly responsible for PA accumulation [7–9]. DG kinase is one of the principal metabolic enzymes of DGs. DG kinases were initially purified from the cytosolic fractions of pig brain [10] and rat liver [11] and had molecular masses of approximately 80 and 120 kDa, respectively, suggesting that there might be different isozymes in various types of cells [12–15]. Sakane *et al.* have cloned the cDNA for the 80 kDa DG kinase and have shown that the primary structure of this isozyme contains EF-hand motifs, typical of calmodulin and other calcium-binding proteins, in addition to zinc finger-like sequences similar to those in PKC [16]. Therefore, calcium and/or phospholipids such as PtdSer were used as direct activators in the purified DG kinase assay system [10, 17]. However, much remains unknown about DG kinase, such as the regulation mechanisms of the enzyme in intact tissues. One reason for this lack of understanding concerning DG kinase regulation mechanisms is that radiolabeled DG may not be used in intact tissues, because endogenous DGs do not penetrate the cell membrane. Moreover, PA is made from alternative pathways, such as hydrolysis of phosphatidylcholine by phospholipase D [18–20]. Thus, DG kinase activity cannot be measured only by the accumulation of PA.

We have previously reported that cell-permeable short chain DG [21, 22] can be useful as an extracellular substrate to measure DG kinase activity in intact tissue (tissue DG kinase assay system) [23]. This exogenous DG substrate inhibited binding of endogenous DG to DG kinase competitively in guinea pig taenia coli, suggesting that the exogenously added diC8 could be phosphorylated by the same isozyme which could then convert endogenous DG to

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Abbreviations: 18:0/20:4-DG, 1-stearoyl-2-arachidonyl-sn-glycerol; 18:0/20:4-PA, 1-stearoyl-2-arachidonyl-phosphatidic acid; BPA, benzylphosphonic acid; BPA/AM, BPA bis-acetoxymethyl ester; CCh, carbachol; DG, diacylglycerol; DG kinase, diacylglycerol kinase; diC8, dioctanoyl-*sn*-glycerol; diC8-PA, dioctanoylphosphatidic acid; DTT, dithiothreitol; fura-2, 1-(2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy)-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; fura-2/AM, fura-2 penta-acetoxymethyl ester; PA, phosphatidic acid; PDBu, phorbol-12,13-dibutyrate; [^{32}P]Pi, radioactive inorganic phosphate; PKC, protein kinase C; PtdSer, L-α-phosphatidyl-L-serine.

Received 24 July 1996; accepted 20 December 1996.

PA. Thus, diC8 is useful as a probe with which to measure DG kinase activity. In this assay system, CCh induced DG kinase is activated in a time- and dose-dependent manner in guinea pig taenia coli. In addition, this activation was mediated by muscarinic receptor stimulation and inhibited by the removal of extracellular calcium. Moreover, phorbol ester also activated DG kinase in tissue with high calcium levels. Therefore, we postulated that muscarinic receptor-mediated activation of DG kinase requires both an increase in intracellular calcium and PKC activation.

We also examined DG kinase distribution in guinea pig taenia coli [24]. Membrane, cytosolic and other fractions were prepared from fresh tissues under various conditions and crude DG kinase was extracted from each fraction. These enzyme samples were added to the mixed micellar assay, and the levels of DG kinase in each fraction were determined. These experiments revealed that DG kinase might be translocated from the cytosol to the membrane by CCh-stimulation, suggesting that DG kinase translocation plays an important role in CCh-induced cellular DG kinase activation in guinea pig taenia coli. However, the activation mechanisms of DG kinase by PKC have yet to be elucidated.

In this paper, we describe the regulation mechanisms of CCh-induced DG kinase translocation in guinea pig taenia coli. We propose that DG kinase is phosphorylated by PKC in the cytosolic area and that the phosphorylated DG kinase migrates to the membrane.

MATERIALS AND METHODS

Materials

Carrier- and HCl-free [^{32}P]Pi and [γ-^{32}P]ATP (111.0 GBq/μmol were purchased from Du Pont-New England Nuclear (Boston, MA, U.S.A.). Octylglycoside, CCh, PtdSer from bovine brain, leupeptin, trypsin inhibitor from soy bean (Type 1-S) and BSA were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). DiC8 and 18:0/20:4-DG were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.). TLC plates (silica gel 60 with a concentrating zone) were obtained from Merck Inc. (Darmstadt, Germany). Okadaic acid, PKC purified enzyme from rat brain and DTT were obtained from Seikagaku Co. (Tokyo, Japan). Fura-2/AM was obtained from Funakoshi Co. (Tokyo, Japan). All other chemicals and materials were of reagent grade.

Isolation and Reagent Treatment of Guinea Pig Taenia Coli

Taenia coli were isolated from guinea pigs (200–300 g body weight, either sex). Tissue specimens, 10–15 mg wet weight tissue, were equilibrated in HEPES-buffered physiological salt solution (Tyrode solution) consisting of 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.6 mM glucose and 8.4 mM HEPES (pH7.4) at 37°C. Tissues were treated with various compounds in 3 mL of Tyrode solution.

Subcellular Fractionation of Guinea Pig Taenia Coli

Tissues treated under the various conditions were homogenized with a polytron homogenizer (Iuchi Co., Japan) in 3 mL of ice-cold solution consisting of 20 mM MOPS (pH 7.2), 250 mM sucrose, 1 mM DTT, 1 mM EGTA, 1 μg/mL pepstatin, 1 μg/mL leupeptin and 50 μg/mL trypsin inhibitor (Buffer A). The homogenates were centrifuged (1,000 × *g* for 5 min) to remove the nuclei. The supernatant was decanted and the pellets washed once with buffer B (sucrose-free buffer A). The combined supernatants were centrifuged again (2,000 × *g* for 30 min). Finally, the membrane fractions were collected by centrifugation (100,000 × *g* for 60 min) and the supernatant was used as the cytosolic fraction.

Crude DG Kinase Preparation and Measurement of DG Kinase Content in the Mixed Micellar Assay

Octylglycoside and KCl were added to the resuspended subcellular fractions on ice to a final concentration of 2% and 300 mM, respectively [25]. The mixtures were slowly shaken for 30 min at room temperature and centrifuged at 100,000 × *g* for 60 min. The supernatants were used in the mixed micellar assay as a crude DG kinase fraction. DG kinase activity was assayed by determining the radioactivity of [^{32}P] incorporated into diC8 or 18:0/20:4-PA. The precursors were dissolved in chloroform and stored at −10°C as a stock solution. Before use, the stock solution was dried under N_2 gas at room temperature and the residue was dissolved in a reaction mixture consisting of 60 mM MOPS (pH 7.2), 0.86 mM DTT, 18 mM $MgCl_2$, 73 mM octylglycoside, 3.3 mM PtdSer and 0.52 mM [γ-^{32}P]ATP (711 GBq/mol). The extracted enzyme or cytosolic fraction was incubated for 5 min at 37°C in the reaction mixture (100 μL). The reaction was terminated by mixing with ice-cold chloroform/methanol/10 M HCl(100:200:1, v/v/v) and the products, diC8-PA and 18:0/20:4-DG, were extracted and separated as described below. Substrate concentrations are expressed as mol% of octylglycoside in the micellar form, and calculations were based on a critical concentration of 25 mM for octylglycoside.

Determination of DG Kinase Activity Using diC8 in Intact Tissue (Tissue DG Kinase Assay)

DG kinase activity in intact whole tissue was determined by measuring the accumulation of [^{32}P]diC8-PA in guinea pig taenia coli prelabeled with [^{32}P]Pi and diC8. Because the [γ-^{32}P]ATP could not penetrate to the cell membrane, [^{32}P]Pi was used in this tissue DG kinase assay. The endogenous PA level was also determined simultaneously by measuring the accumulated [^{32}P]PA in each tissue. In this assay, the diC8 stock solution was dried under N_2 gas at room temperature and dissolved in 50% ethanol (final 0.03%). The diC8 ethanol mixture was added to Tyrode solution containing 2.7 mg/mL BSA (diC8 solution). The

fresh tissues were incubated with 2.22 MBq/mL of [^{32}P]Pi in 1 mL of diC8 solution for 30 min at 37°C and washed twice with 10 mL Tyrode solution. The reaction was then started by adding 0.8 mL of Tyrode solution containing various compounds. The reaction was terminated by mixing with 3 mL of ice-cold chloroform/methanol/10 M HCl (100:200:1, v/v/v), and the tissues were then homogenized using a glass homogenizer in ice-cold water. [^{32}P]diC8-PA and [^{32}P]PA were extracted as described below.

Extraction and Analysis of [^{32}P]diC8-PA, [^{32}P]18:0/20:4-PA and [^{32}P]PA

One milliliter each of chloroform and 0.1 M HCl were added to the reaction mixture and the mixture was vigorously shaken followed by centrifugation for 20 min at 1,000 × *g* to allow phase separation [26]. The lower phase was carefully removed and dried under N_2 gas at room temperature. The residue was then redissolved in 40 μL of chloroform and spotted on TLC plates, which were developed using ethylacetate/isooctane/acetic acid/water (7:5:2:1, v/v/v/v) [22], to separate diC8-PA (Rf 0.27), 18:0/20:4-PA and endogenous PA (Rf 0.51) from each other and from major phospholipids. The spots corresponding to [^{32}P]diC8-PA and [^{32}P]18:0/20:4-PA localized by autoradiography (−20°C, overnight) were scraped and the radioactivity level was counted by means of liquid scintillation spectrometry.

PKC Treatment of DG Kinase Samples for Measurements of DG Kinase Affinity

Purified PKC was solubilized to a final concentration of 0.1 μg/mL in 60 mM MOPS (pH 7.2), 0.86 mM DTT, 18 mM $MgCl_2$, 73 mM octylglycoside, 3.3 mM PtdSer, 20 μMg/mL leupeptin, 1 mM EGTA, 0.875 mM $CaCl_2$ (1 μM free) and 0.52 mM ATP. Solubilized and extracted DG kinase from membrane and cytosolic fractions were added. Following preincubation at 37°C, the reaction was initiated by addition of octylglycoside mixed micelles. After 2 min incubation, the reaction was terminated and centrifuged at 100,000 × *g* for 60 min to separate mixed micelles from the attached DG kinase. The products in the mixed micelles pellet, diC8-PA and 18:0/20:4-PA, were detected as described for the mixed micellar assay.

Fura-2 Loading for Measuring Intracellular Calcium Concentration and Force Development

Fresh tissues were preincubated with 5 μM fura-2/AM in Tyrode solution containing the cremophore EL (final 0.06%). Loading proceeded at 18°C for 6 hr [27], after which the tissues were transferred to Tyrode solution. These experiments were performed within 30 min after rising, and changes in fluorescence intensity were monitored using a fluorometer specially designed to measure surface fluorescence from living tissue (CAF-100, Japan Spectroscopic, Tokyo, Japan). The ratio of the fluorescence due to excitation at 340 nm to that at 380 nm ($R_{340/380}$) was calculated from successive illumination periods. The tissue was held horizontally in a temperature-controlled, 3 mL organ bath. One end of the tissue was connected to a strain gauge transducer (Type T-7-8-240, Orientec, Tokyo, Japan) to monitor mechanical activity.

Other Assays

Protein concentrations were determined by Bio-Rad protein assay reagent using BSA as the standard. PKC activity was determined by an Amersham protein kinase C assay kit.

Data Analysis

Data are shown as means ± S.E. of the values obtained from at least five separate animals. The significance of the difference between the values was assessed by the one-way analysis of variance (ANOVA) followed by *Bonferroni t*-test for multiple comparisons.

RESULTS

Changes in the DG Kinase Distribution Induced by CCh

The time course of DG kinase translocation in guinea pig taenia coli after treatment with 100 μM CCh was determined in the membrane and cytosolic fractions (Fig. 1). In this system, two species of DGs were used as substrates: 18:0/20:4-DG as typical endogenous DG [28] and diC8 as a common substrate in the tissue DG kinase assay system. The phosphorylation rates of both substrates in each fraction were determined simultaneously.

In the presence of 100 μM CCh, the rate of phosphorylation in the membrane fraction increased rapidly in the first 3 min, with a maximal increase detected after 5 min (262% of the non-treated control). This CCh-induced increase was sustained over the remainder of the 10 min incubation period and by 30 min after stimulation, the rates of diC8 phosphorylation in the membrane fraction had gradually decreased to 180% of the non-stimulated control (data not shown). The rate of diC8 phosphorylation in the cytosolic fraction decreased in the first 3 min of the incubation, with a maximal decrease detected at 5 min (9.5% of the non-treated control). This decrease was sustained for 30 min. The CCh-induced changes in diC8 phosphorylation rates in both the membrane and cytosolic fractions were complementary. Incubating tissues without CCh did not alter the phosphorylation rates of diC8 in the membrane and cytosolic fractions. Changes in the total content of DG kinase in both the membrane and cytosolic fractions by CCh stimulation were also examined. Tissues were incubated in the presence or absence of 100 μM CCh and subcellular fractions were then collected. Supernatant of 20,000 × *g* centrifugation at 4°C for 30 min which contained both the membrane and cytosolic fractions were

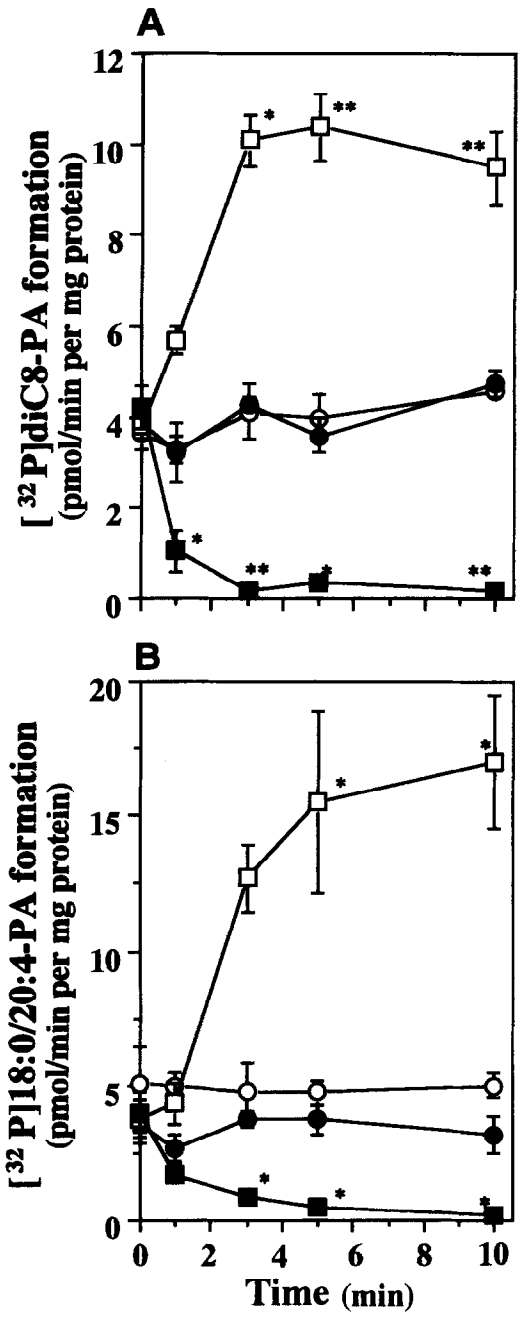

FIG. 1. Time-course of CCh-induced change in DG kinase distribution in guinea pig taenia coli. After guinea pig taenia coli were incubated in the presence (squares) or absence (circles) of 100 μM CCh at 37°C for the indicated periods, the tissues were homogenized and each fraction collected. DiC8 and 18:0/10:4-DG stock solutions were dried under N_2 gas and dispersed in octylglycoside micelles consisting of 60 mM MOPS, 0.86 mM DTT, 18 mM $MgCl_2$, 73 mM octylglycoside, 3.3 mM PtdSer and 0.52 mM ATP. Extracted crude DG kinase from each fraction was incubated with the mixed micelles for 5 min at 37°C, and the rates of phosphorylation of diC8 (A) and 18:0/10:4-DG (B) by DG kinase prepared from the membrane (open) and cytosolic (closed) fractions were measured as described in Materials and Methods. *$P < 0.05$, **$P < 0.01$ values significantly different from the non-stimulated control.

collected from each tissue. DG kinase samples were extracted and the contents measured by mixed micellar assay. Phosphorylation of diC8 was 4.7 ± 0.4 pmol/min per mg protein at 1 min incubation of non-stimulated tissue. This value did not change during the incubation. In the CCh-stimulated tissue, the phosphorylation rate of diC8 was 4.3 ± 0.7 pmol/min per mg protein at 1 min incubation, a value which also remained constant during the incubation. These values were not significantly different from those in the non-stimulated control (data not shown). The 18:0/20:4-DG phosphorylation rates were similar to those of the diC8 phosphorylation rates. Non-stimulated control levels of [^{32}P]PA at 5 min in the membrane and cytosolic fractions were 4.7 ± 0.5 and 3.8 ± 0.6 pmol/min per mg protein, respectively. These values remained the same throughout the 10 min incubation period. Treatment of 100 mM CCh induced an increase in [^{32}P]PA formation in the membrane fraction, while [^{32}P]PA formation was decreased by the treatment in the cytosolic fraction. These changes were sustained over the 10 min incubation. These values in the membrane and cytosolic fractions at 5 min were 15.5 ± 3.4 and 0.5 ± 0.2 pmol/min per mg protein, respectively.

Effects of Intracellular Calcium Concentration on Total Cellular Activity and the Intracellular Distribution of DG Kinase

To determine the effect of the intracellular calcium concentration on DG kinase activity and its distribution, experiments were performed under the two conditions. First, the CCh-induced calcium influx from extracellular medium was reduced by eliminating extracellular calcium (Ca^{2+}-free Tyrode solution) [29] and second, the tissues were incubated in Ca^{2+}-free Tyrode solution containing 5 mM EGTA (EGTA-Tyrode solution) to reduce both intracellular and extracellular calcium. Under these conditions, changes in intracellular calcium concentration and force development induced by CCh were examined.

Fig. 2A shows typical records of force development determined isometrically and changes in the $R_{340/380}$ indicating the intracellular calcium concentration of the fura-2 loaded tissue. When the extracellular calcium level was at a physiological concentration (1.8 mM Ca^{2+}: normal Tyrode solution), 100 μM CCh induced a significant and transient contraction (phasic component) followed by a smaller and sustained contraction (tonic component). In fura-2 loaded preparations, addition of CCh rapidly increased the $R_{340/380}$, indicating an increase in the intracellular calcium concentration. The $R_{340/380}$ gradually decreased during the tonic component. $R_{340/380}$ and force development were measured in the absence of diC8. However, a preliminary study was conducted to ensure that preincubation of tissue with 100 μM diC8 at 37°C for 60 min had no effect on the CCh-induced increase in $R_{340/380}$ or force development. Moreover, preincubation of tissue with 0.06% cremophore EL did not influence either force

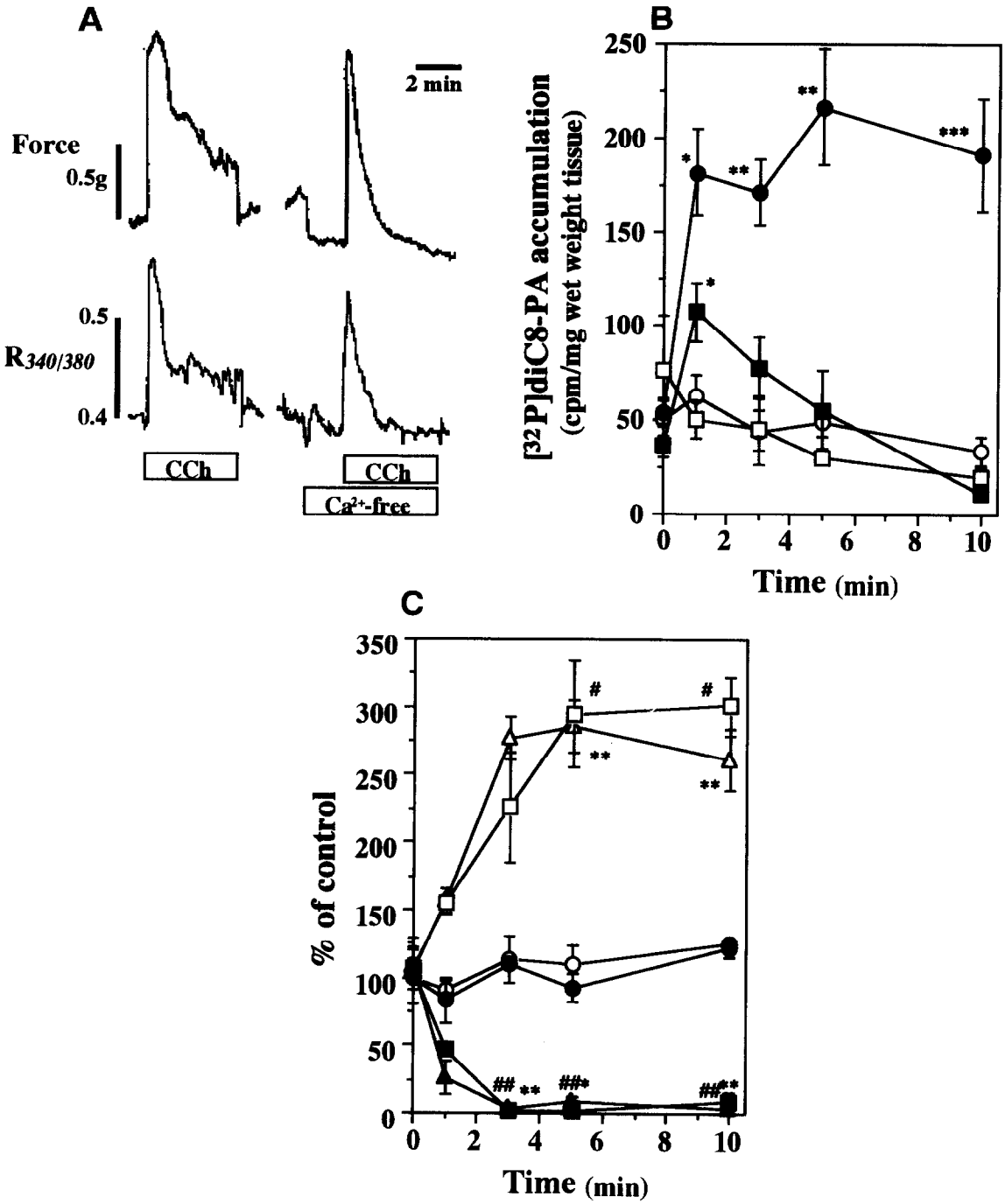

FIG. 2. Effects of calcium release from the intracellular stores on CCh-induced cellular DG kinase activation and distribution. In fura-2 loaded guinea pig taenia coli, force development (upper trace) and $R_{340/380}$ (lower trace) induced by 100 μM CCh were simultaneously measured (A). Extracellular calcium was removed 2 min before stimulation. Total cellular DG kinase activity was measured by means of the tissue DG kinase assay. Fresh tissues were incubated with 2220 GBq/mL [^{32}P]Pi and 100 μM diC8 at 37°C for 30 min (B). After washing, these tissues were incubated in the presence (closed) or absence (open) of 100 μM CCh at 37°C. The extracellular medium was changed to Tyrode solution (circles) or Ca^{2+}-free Tyrode solution (squares) 2 min before stimulation. [^{32}P]diC8-PA was quantified in these tissues as described in Materials and Methods. Each value represents the mean ± SE of at least five independent determinations. The distribution of DG kinase was measured by means of the mixed micellar assay. Fresh tissues were incubated in the presence (triangles, squares) or absence (circles) of 100 μM CCh at 37°C for the indicated periods (C). The extracellular medium was changed from normal Tyrode solution (circles, triangles) to Ca^{2+}-free Tyrode solution (squares) under the same conditions as described in (B). The rates of diC8 phosphorylation in the membrane (open) and cytosolic fractions (closed) were measured as described in Materials and Methods. Results are expressed as a percent of the non-treated control at 0 min. *,#$P < 0.05$; **,##$P < 0.01$; ***,###$P < 0.001$ values significantly different from each fraction of the control.

development or PKC activity under these conditions (data not shown).

When extracellular calcium was removed by replacing the medium with Ca^{2+}-free Tyrode solution at 2 min before stimulation, CCh-induced force development and $R_{340/380}$ were reduced. The magnitudes of inhibition in the tonic phase of $R_{340/380}$ and force development were 99 and 97%, respectively in 5 independent examinations (data not shown). However, the phasic and tonic components were reduced by only 11 and 4.8%, respectively.

To determine cellular DG kinase activity under the same conditions as force development, we measured the time-course of accumulation of [^{32}P]diC8-PA and [^{32}P]PA in [^{32}P]Pi- and diC8-prelabeled guinea pig taenia coli in normal Tyrode solution (Fig. 2B). In this assay, diC8 was used as an exogenous substrate of DG kinase, although, some reports have suggested that diC8 increases PKC in some types of cells. Therefore, we investigated the effects of diC8 incorporation on PKC activity. Cellular PKC activity was not affected by incorporation of diC8 as a exogenous substrate under our experimental conditions (data not shown). [^{32}P]diC8-PA levels were not increased in the absence of CCh (control). In the presence of 100 μM CCh, the accumulation of [^{32}P]diC8-PA significantly increased (from a basal value of 62.0 ± 11.2 to 182.9 ± 23.8 cpm/mg wet weight tissue at 5 min incubation) and was sustained over the remainder of the 30 min incubation period. In the Ca^{2+}-free Tyrode solution, the [^{32}P]diC8-PA level without CCh stimulation was the same as that in normal Tyrode solution, while 100 μM CCh caused a transient increase in [^{32}P]diC8-PA accumulation. The peak value was 107.5 ± 16.8 cpm/mg wet weight tissue at 1 min after CCh-stimulation and 59% of the peak value of the normal Tyrode solution. [^{32}P]diC8-PA accumulation returned to the control level at 5 min after stimulation. [^{32}P]PA accumulation corresponded well to the changes in [^{32}P]diC8-PA levels. In the normal Tyrode solution, treatment with 100 μM CCh induced a sustained increase in [^{32}P]PA level (from non-stimulated control levels of 533.4 ± 63.4 to 1396.1 ± 144.2 cpm/mg wet weight tissue at 5 min after stimulation). In the Ca^{2+}-free Tyrode solution, treatment with CCh induced a transient increase in [^{32}P]PA (the peak value was 1281.0 ± 211.7 cpm/mg wet weight tissue at 1 min after CCh stimulation). This [^{32}P]PA level returned to the control level at 5 min after stimulation.

The distribution of DG kinase under the same conditions was also measured (Fig. 2C). In Ca^{2+}-free Tyrode solution, control rates of diC8 phosphorylation in both fractions were the same as those of non-stimulated tissue in the normal Tyrode solution. These values in the membrane and cytosolic fractions at 5 min were 4.1 ± 0.4 and 3.9 ± 0.2 pmol/min per mg protein, respectively. CCh increased the rate of phosphorylation in the membrane fraction, while the phosphorylation rate decreased in the cytosolic fraction without extracellular calcium. Significant changes in the phosphorylation rates of diC8 in the membrane and cytosolic fractions were detected at 5 min after stimulation. The phosphorylation rates of diC8 in the membrane and cytosolic fractions were 10.7 ± 1.4 and 0.1 ± 0.1 pmol/min per mg protein, respectively. The time course of the CCh-induced changes in the rates of phosphorylation in Ca^{2+}-free Tyrode solution was similar to that in the normal Tyrode solution. These CCh-induced changes were the same as those for 18:0/20:4-DG phosphorylation (data not shown).

To investigate the effects of reducing intracellular and extracellular calcium by EGTA-Tyrode solution on cellular DG kinase activity and the distribution, CCh-induced changes in intracellular calcium concentration and force development were measured (Fig. 3A). The CCh-induced biphasic increase in force development and the $R_{340/380}$ was completely abolished without affecting the control levels by replacing the medium with EGTA Tyrode solution 5 min before stimulation. The basal levels of [^{32}P]diC8-PA in non-stimulated tissue in EGTA- and normal Tyrode solutions were the same (31.9 ± 15.8 cpm/mg wet weight tissue at 5 min incubation) (Fig. 3B). CCh did not change [^{32}P]diC8-PA accumulation in EGTA-Tyrode solution. [^{32}P]PA accumulation corresponded in [^{32}P]diC8-PA level. Treatment with 100 μM CCh did not induce changes in the [^{32}P]PA level in the EGTA-Tyrode solution (from non-stimulated control levels of 564.8 ± 53.4 to 528.2 ± 84.1 cpm/mg wet weight tissue at 5 min after the stimulation). The distribution of DG kinase under the same conditions was also detected (Fig. 3C). Control phosphorylation rates of diC8 in both the membrane and cytosolic fractions were the same as the values in non-stimulated tissue in normal Tyrode solution, i.e., 4.7 ± 0.1 and 3.2 ± 0.6 cpm/mg wet weight tissue at 5 min incubation, respectively. CCh did not affect the phosphorylation rates of diC8 in the membrane and cytosolic fractions (4.0 ± 0.5 and 3.8 ± 0.7 cpm/mg wet weight tissue at 5 min incubation, respectively). Replacing the extracellular medium by EGTA-Tyrode solution at 5 min before stimulation did not change cellular DG kinase activity nor DG kinase distribution.

Effect of PKC on DG Kinase Affinities

To understand how the distribution of DG kinase is regulated by PKC, we examined the effect of DG kinase phosphorylation by PKC on DG kinase affinities to the octylglycoside mixed micelles (Fig. 4). Crude DG kinases were prepared from the membrane and cytosolic fractions. These enzyme samples were preincubated in the presence or absence of 0.1 μg/mL purified PKC for various periods at 37°C, and the rates of phosphorylation of diC8 and 18:0/20:4-DG by mixed micelles attached DG kinase samples were then measured by means of the mixed micellar assay. The phosphorylation rates of diC8 did not increase in the absence of PKC over a 10 min incubation period. The values in the membrane and cytosolic fractions were 4.3 ± 0.6, 2.6 ± 0.4 pmol/min per mg protein, respectively. In the

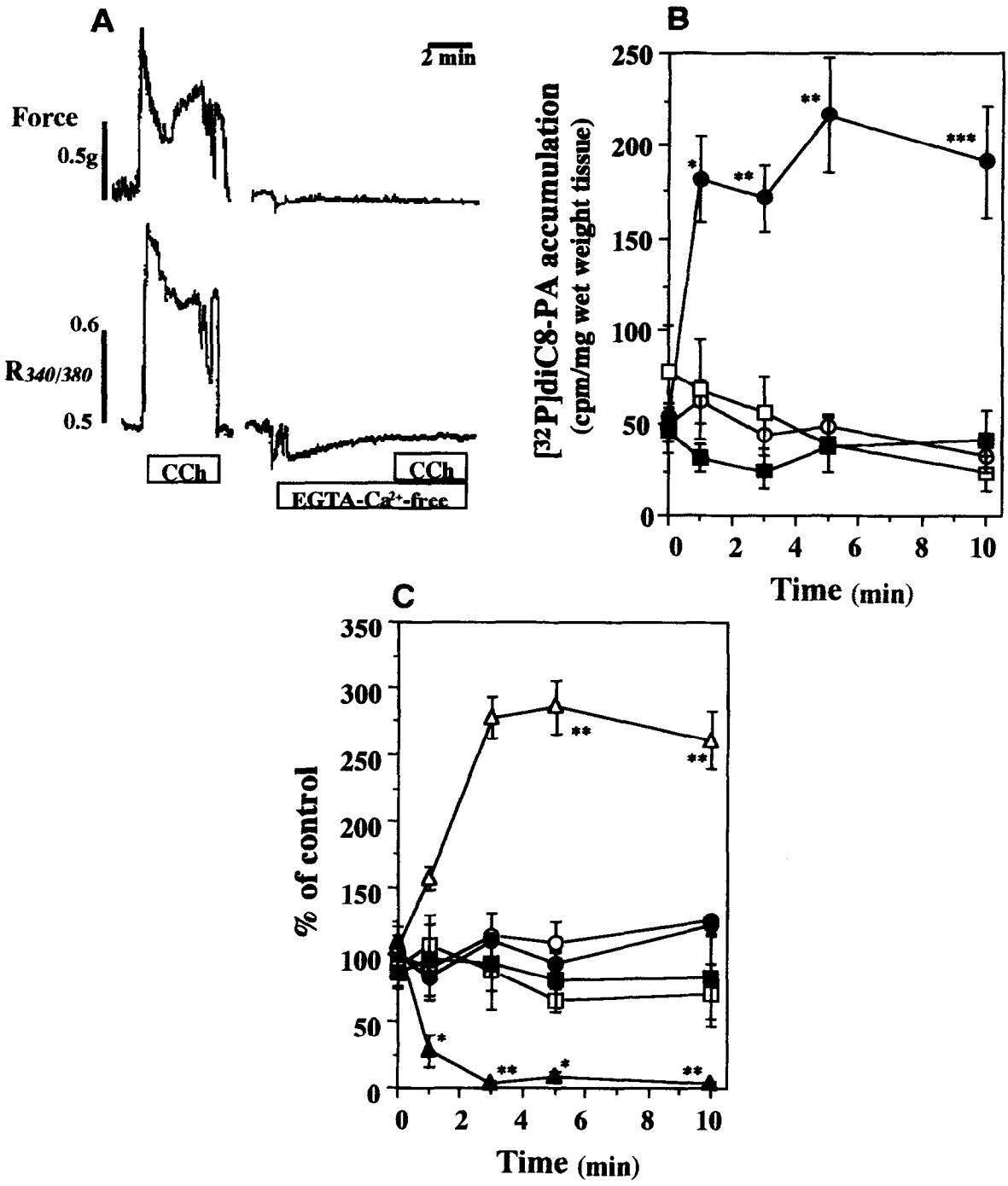

FIG. 3. Effects of intracellular calcium deletion on CCh-induced DG kinase activation and distribution. Force development (upper trace) and $R_{340/380}$ (lower trace) were measured in fura-2 loaded guinea pig taenia coli (A). The extracellular medium was changed to EGTA-Tyrode solution 5 min before stimulation. Total cellular DG kinase activity was measured by means of the tissue DG kinase assay. Tissues that incorporated [^{32}P]Pi and diC8 were incubated in the presence (closed) or absence (open) of 100 μM CCh at 37°C (B). The extracellular medium was changed to Tyrode solution (circles) or EGTA-Tyrode solution (squares) 5 min before stimulation. The [^{32}P]diC8-PA levels were quantified as described in Materials and Methods. Each value represents the mean ± SE of at least five independent determinations. The distribution of DG kinase was measured using the mixed micellar assay. Fresh tissues were incubated in the presence (triangles, squares) or absence (circles) of 100 μM CCh at 37°C for the indicated periods (C). The extracellular calcium was changed to Tyrode solution (circles, triangles) or EGTA-Tyrode solution (squares) 5 min before stimulation (B). These tissues were homogenized and fractionated. The phosphorylation rates of diC8 in the membrane (open) and cytosolic fractions (closed) were measured as described in Materials and Methods. The results are expressed as a percent of the non-treated control at 0 min. *,#P < 0.05; **,##P < 0.01, ***,###P < 0.001 values significantly different from each fraction of the control.

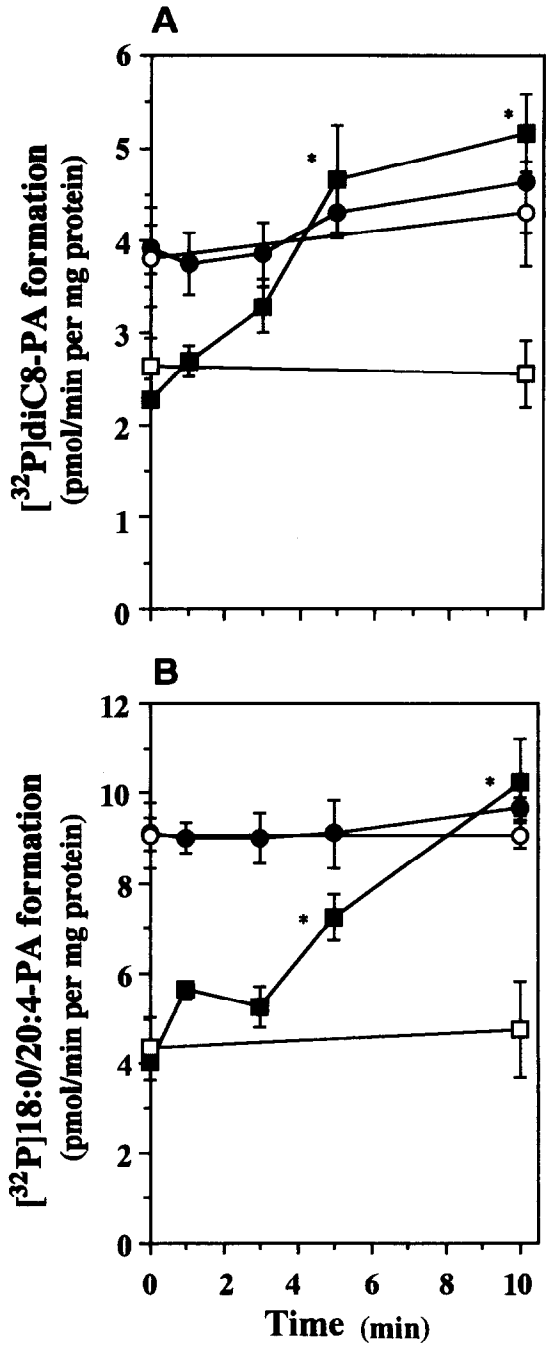

FIG. 4. Effects of DG kinase phosphorylation by PKC. Guinea pig taenia coli were homogenized and subcellular fractions were collected. Extracted crude DG kinase from the membrane (circles) and cytosolic fractions (squares) were incubated in the presence (closed) or absence (open) of 0.1 mg/mL PKC solution consisting of 60 mM MOPS (pH 7.2), 0.86 mM DTT, 18 mM $MgCl_2$, 73 mM octylglycoside, 3.3 mM PtdSer, 20 μMg/mL leupeptin, 1 mM EGTA, 0.875 mM $CaCl_2$ (1 μM free) and 0.52 mM ATP at 37°C for the indicated periods. Pretreated crude DG kinases were incubated with the mixed micelles for 2 min at 37°C. Then, mixed micelles with attached DG kinase were collected by centrifugation (100,000 × g, 4°C, 60 min), and the phosphorylation rates of diC8 (A) and 18:0/20:4-DG (B) measured as described in Materials and Methods. Each value represents the mean ± SE of at least five independent determinations. *P < 0.05 values significantly different from those of controls at 0 min.

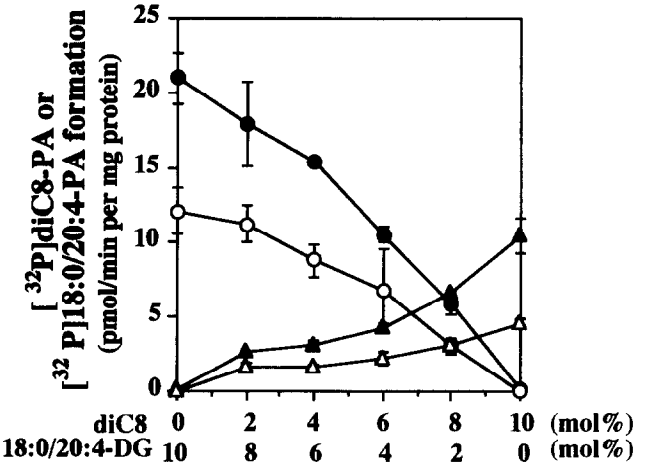

FIG. 5. Competition between diC8 and 18:0/20:4-DG for phosphorylation by crude DG kinase exposed to PKC. Guinea pig taenia coli were homogenized and cytosolic fractions (100,000 × g, supernatant) were collected. Crude DG kinase extracted from this fraction was incubated in the presence (open) or absence (closed) of 0.1 μM/mL PKC at 37°C for 10 min under the same conditions as described in Fig. 4. Mixtures of diC8 and 18:0/20:4-DG were dried under N_2 gas and dispersed in octylglycoside micelles. The total amount of the two species of DGs was kept at 10 mol% during the incubation. DG mixtures in each combination were incubated with pretreated crude DG kinase as described in Materials and Methods. The formation of [32P]diC8-PA (triangles) and [32P]18:0/20:4-PA (circles) was quantified as described in Materials and Methods. Each value represents the mean ± SE of at least 5 independent determinations.

presence of PKC, the rate of phosphorylation of diC8 by DG kinase prepared from the membrane fraction was not different from that in the absence of PKC. On the other hand, the rate of phosphorylation of diC8 by DG kinase prepared from the cytosolic fraction increased significantly after a 5 min incubation with standard PKC, reaching a maximal 2-fold increase over the basal values (at 5.2 ± 0.4 pmol/min per mg protein) after a 10 min incubation. At 10 min, the phosphorylation rate of diC8 by PKC-pretreated DG kinase extracted from the cytosolic fraction was similar to that in the non-treated DG kinase prepared from the membrane fraction. Similarly, the rate of phosphorylation of 18:0/20:4-DG was increased only by PKC-treated DG kinase extracted from the cytosolic fraction, the maximal levels being 2.5-fold greater than the non-treated control level (10.3 ± 0.9 pmol/min per mg protein).

To investigate whether the PKC affects the substrate selectivity of DG kinase or not, we compared substrate selectivity between PKC-treated and non-treated DG kinase extracted from the cytosolic fraction (Fig. 5). Crude DG kinase was extracted from the cytosolic fraction and incubated with or without PKC at 37°C for 10 min. Then, mixed micelles were prepared in which the proportions to the two substrates were varied, while the total DG concentration was kept constant at 10 mol%. In the crude DG kinase without PKC, the rate of phosphorylation of 18:0/20:4-DG decreased sharply, while that of diC8 phosphory-

lation increased. The phosphorylation rates of both species were the same in mixed micelles, with a 4:1 ratio of diC8 to 18:0/20:4-DG (8 mol% of diC8 and 2 mol% of 18:0/20:4-DG). In a mixture containing equal amounts (5 mol%) of each specimen, the rate of phosphorylation of 18:0/20:4-DG was 4-fold higher than that of diC8. With the PKC treated DG kinase, although the phosphorylation rates of diC8 and 18:0/20:4-DG increased, these rates for both species were equal in mixed micelles, with a 4:1 ratio of diC8 to 18:0/20:4-DG. These values agreed with the rates for the non-treated DG kinase.

Effect of Phosphatase Inhibitor on CCh-induced Changes in the DG Kinase Distribution

We examined whether or not CCh-induced changes in DG kinase distribution were irreversible and mediated by DG kinase phosphorylation, using the serine/threonine phosphatase-1 and -2A inhibitor, okadaic acid [30, 31] and the tyrosine phosphatase inhibitor, BPA [32] (Fig. 6). Because the BPA could not penetrate to the cell membrane, it was treated as bis-acetoxymethyl ester (BPA/AM). Fresh tissues were incubated with 100 μM CCh at 37°C for 5 min and the membrane and cytosolic fractions were collected. Crude DG kinase was extracted from both fractions for use in the mixed micellar assay system. The phosphorylation rates of diC8 were significantly increased by CCh treatment in the membrane fraction with a concomitant decrease in the cytosolic fraction, i.e., 9.9 ± 1.8 and 1.3 ± 0.1 pmol/min per mg protein, respectively. When tissues were incubated with 100 μM CCh at 37°C for 5 min and then washed four times with Tyrode solution, CCh-induced changes in the rates of phosphorylation of diC8 in both fractions returned to control levels (4.1 ± 0.3 and 4.2 ± 0.3 pmol/min per mg protein in the membrane and cytosolic fractions, respectively). When okadaic acid (10 μM) was added 5 min before CCh stimulation, CCh induced significant changes in the phosphorylation rates of diC8 in both fractions. These values in the membrane and cytosolic fractions were 9.0 ± 0.6 and 1.2 ± 0.1 pmol/min per mg protein, respectively. When CCh-stimulated tissue was washed in the presence of okadaic acid with Tyrode solution containing 10 μM okadaic acid, the CCh-induced changes in the rates of phosphorylation of diC8 did not return to control levels in either fraction (7.0 ± 1.2 and 1.4 ± 0.1 pmol/min per mg protein, respectively). On the other hand, preincubation of tissue with 10 μM BPA/AM 15 min before 100 μM CCh stimulation was not affected by CCh-induced changes in the diC8 phosphorylation rate in either the membrane or cytosolic fractions, i.e., 8.8 ± 0.9 and 1.0 ± 0.1 pmol/min per mg protein, respectively. Moreover, when CCh-stimulated tissue was washed in the presence of BPA with Tyrode solution containing 10 μM BPA/AM, CCh-induced changes in the rates of phosphorylation of diC8 in both fractions returned to control values (3.3 ± 0.6 and 3.2 ± 0.1 pmol/min per mg protein, respectively). The

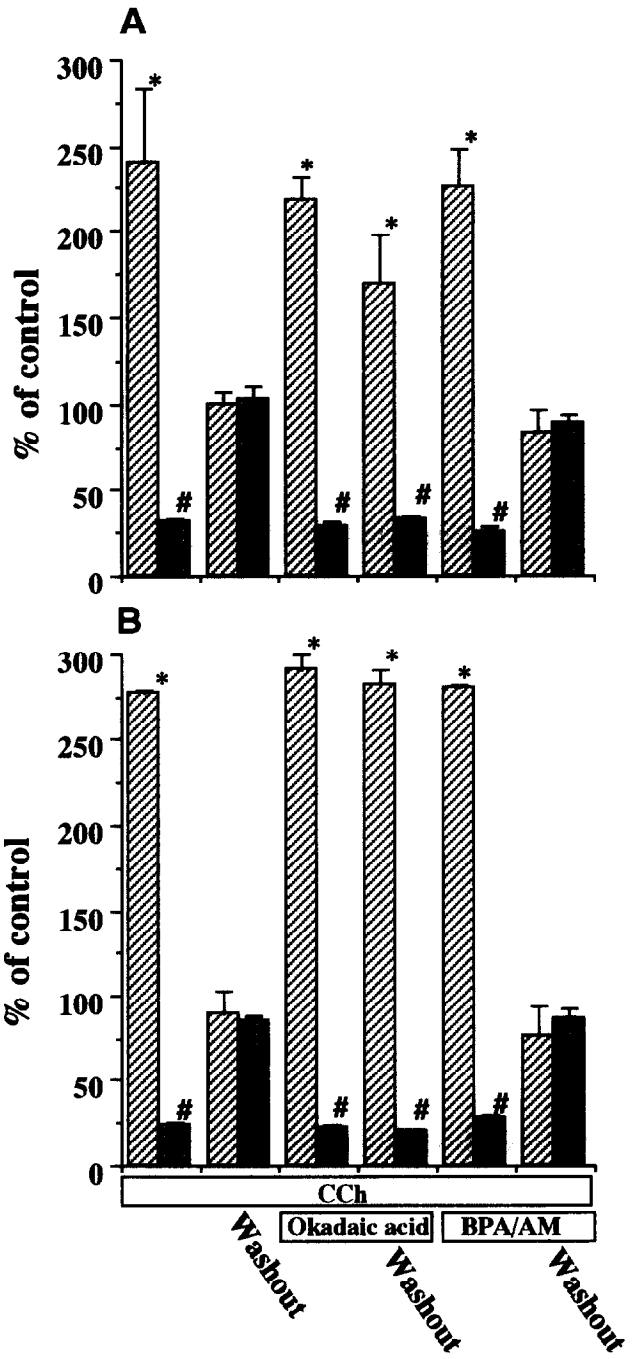

FIG. 6. Effect of phosphatase inhibitor on the CCh-induced change in DG kinase distribution. Fresh tissues were incubated in the presence or absence of 100 μM CCh at 37°C for 5 min. Some tissues were then washed four times with Tyrode solution (washout). Okadaic acid (10 μM) was added 5 min before CCh stimulation. BPA was added as acetoxymethyl ester (BPA/AM, 10 μM) 30 min before CCh stimulation. The tissues were then homogenized and fractionated. The phosphorylation rates of diC8 (A) and 18:0/20:4-DG (B) in the membrane (▨) and cytosolic fractions (■) were measured as described in Materials and Methods. The results are expressed as a percent of the non-treated control. *,#$P < 0.05$ values significantly different from those of control membrane and cytosolic fractions.

18:0/20:4-DG phosphorylation rates were similar to those of the diC8 phosphorylation rates.

DISCUSSION

We showed previously that DG kinase is activated by CCh in a time- and dose-dependent manner in guinea pig taenia coli [23], and that this DG kinase activation depended both on an increase in intracellular calcium concentration as well as PKC activation. We also demonstrated that DG kinase activity existed mainly in the cytosolic fraction at the resting state, while the activity translocated to the membrane fraction after incubation of the tissue with CCh. The properties of this alteration were very similar to those of typical enzyme translocations from the cytosol to the membrane [24]. Maroney and Maraca [33] reported that DG kinase translocation was regulated by changes in membrane-associated DG content in Swiss 3T3 cells. Moreover, van der Bend *et al.* [34] reported that an increase in DG kinase activity in the membrane fraction was evoked by an increase in DG using bacterial phosphatidylinositol-specific phospholipase C in Jurkat cells. These studies suggested that the translocation of DG kinase is regulated by DG content in the plasma membrane. However, from the findings of our experiments that translocation was blocked by PKC inhibitor, we suggest that PKC is involved in the translocation of DG kinase.

We examined how intracellular calcium concentration and PKC activity contributed to the translocation of DG kinase. In our assay system, fresh tissues were incubated in the presence or absence of CCh for various periods, and crude DG kinase was then extracted from the membrane and cytosolic fractions from each tissue for the octylglycoside mixed micellar assay to detect the phosphorylation rates of diC8 and 18:0/20:4-DG. In this system, we added an excess amount of PtdSer as a direct DG kinase activator to octylglycoside micelles [10, 11] containing substrates (diC8 and 18:0/20:4-DG) and ATP to induce full activity of DG kinase in order to determine the content of the enzyme in each fraction. Consequently, the phosphorylation rates of diC8 and 18:0/20:4-DG detected in this assay system do not indicate DG kinase activities but represent the loading of DG kinase in the cells. Thus, an apparent increase in the phosphorylation rate represents an increase in the total content of DG kinase. Using this method, we measured the CCh-induced changes in DG kinase content in the membrane and cytosolic fractions, and investigated the mechanisms of DG kinase translocation. Fig. 1 shows that the rates of phosphorylation of diC8 and 18:0/20:4-DG in the membrane fraction increased, whereas those in the cytosolic fraction decreased during the first 3–5 min of incubation with CCh in a time-dependent manner. These changes were sustained over 10 min. The findings that the total content of DG kinase in membrane and cytosolic fractions did not change during CCh stimulation suggests that such changes in DG kinase distribution might be translocated from the cytosol to the membrane rather than induced in the membrane fraction. Further, these results indicate that the translocation of DG kinase develops rapidly and is completed within 3–5 min of stimulation. The time-course of DG kinase translocation in human neutrophils has been reported [35]. In neutrophils, phorbol ester induced a sustained DG kinase translocation, whereas formylmethionyl-leucyl-phenylalanine induced a transient process. Our results were similar to these findings using phorbol ester.

We examined the effects of intracellular calcium concentration on CCh-induced DG kinase translocation (Figs. 2 and 3). In many types of smooth muscle cells including guinea pig taenia coli, CCh induces a biphasic intracellular calcium increase and force development [29]. This initial transient increase in intracellular calcium concentration depends on calcium release from intracellular stores, whereas the sustained increase depends on calcium influx from the extracellular medium. We examined which source, intracellular stores or extracellular medium, contributes to the development of DG kinase translocation and found that DG kinase translocation was developed by a transient increase in intracellular calcium, probably released only from intracellular calcium stores, because the CCh-induced translocation was normally sustained under calcium influx suppression (Fig. 2C). When the intracellular calcium concentration returned to the basal level after the transient increase induced by CCh, total cellular DG kinase activity also reached the non-stimulated control level, although DG kinase translocation was sustained. These results indicated that DG kinase was translocated to the membrane containing DGs at 5 min in calcium-free medium, but that DG was not phosphorylated. Considering that the calcium dependency of DG kinase has been confirmed using a purified enzyme fraction extracted from many types of cells [16, 22], calcium influx from the extracellular medium might be required for the phosphorylation of DG by translocated DG kinase in the membrane. In EGTA-Tyrode solution, cellular DG kinase activation and translocation were not stimulated by CCh. These results also support such a notion.

We examined the effect of the DG kinase regulator, PKC, on CCh-induced DG kinase translocation. The CCh-induced DG kinase translocation was inhibited by the PKC inhibitor, H-7 [24], while the PKC activator, phorbol ester, induced the translocation in the tissue with the accumulated intracellular calcium. Based on these results, we suggest that PKC is involved in the translocation of DG kinase. In many types of cells, PKC regulates enzyme activity through phosphorylation [2, 4]. Kanoh *et al.* have reported that the DG kinase from pig thymus [36] and brain [37] are phosphorylated *in vitro* by PKC. To determine whether or not the modification of DG kinase by PKC is necessary to initiate translocation of DG kinase from the cytosol to the membrane, DG kinases were incubated with purified PKC and the affinities of DG kinase for the membrane measured. DG kinase samples obtained from CCh-non-treated tissues were incubated with purified PKC

for various periods and the phosphorylation rates of diC8 and 18:0/20:4-DG were measured using the same quantities of these DG kinase samples. DG kinase associated with the mixed micelles with a certain affinity and then phosphorylated diC8 and 18:0/20:4-DG. The period of incubation of DG kinase samples with mixed micelles (2 min) was half the maximal period of diC8 phosphorylation (data not shown). Following incubation, mixed micelles with attached DG kinase and products were separated from solubilized DG kinase, and diC8-PA and 18:0/20:4-PA in mixed micelles were detected. Therefore, the phosphorylation rates indicate the affinity of DG kinase for the mixed micelles rather than the total content of DG kinase in each sample. If PKC does not cause any modification of the enzyme before association with mixed micelles, a similar phosphorylation rate should be obtained for the PKC-treated and non-treated DG kinase. The affinity of DG kinase extracted from the membrane fraction to the mixed micelles was not altered by PKC, though it had a higher affinity in comparison with the DG kinase extracted from the cytosolic fraction. However, the affinity of DG kinase to mixed micelles extracted from the cytosolic fraction increased depending on the exposure period to PKC, reaching a maximum at 10 min in the presence of PKC. These results indicate that the phosphorylation of cytosolic DG kinase by PKC induces the transfer of DG kinase to the membrane. We checked the substrate selectivity of PKC-treated and non-treated DG kinase extracted from the cytosolic fraction (Fig. 5). The rate of phosphorylation of 18:0/20:4-DG was more than 4-fold that of diC8. The phosphorylation rates of 18:0/20:4-DG by cytosolic DG kinase treated with PKC was over 4-fold that of diC8, and the ratio was the same as that of 18:0/20:4-DG. These results indicate that PKC treatment with DG kinase did not change the substrate selectivity of DG kinase. Therefore, we considered that the increase in the rates of phosphorylation of DG induced by PKC-treated DG kinase was not due to a change in the substrate selectivity of the enzyme, but rather to a change in the affinity of DG kinase for the mixed micelles.

We consider that CCh-induced DG kinase translocation is reversible, and that dephosphorylation causes relocation of DG kinase. In this study, we examined serine/threonine phosphatase-1, -2A and tyrosine phosphatase as typical dephosphorylation enzymes, and investigated how the phosphorylation participates in the regulation of DG kinase translocation. We measured the influence of phosphatase inhibition by okadaic acid [30, 31] and BPA/AM [32] on the distribution of DG kinase in the mixed micellar assay under the same conditions as outlined in Fig. 1 (Fig. 6). Basal and CCh-induced changes in DG kinase distribution were not influenced by 10 μM okadaic acid. Okadaic acid inhibited the recovery of DG kinase translocation depending on the removal of CCh. However, the tyrosine phosphatase inhibitor, BPA/AM, did not affect the recovery of DG kinase translocation. These results indicate that DG kinase translocation occurs through serine/threonine phosphorylation and is recovered by dephosphorylation depending on serine/threonine phosphatase. Although the target of the inhibitory effects of okadaic acid could not restrict, serine/threonine phosphorylation of DG kinase by PKC was reported [36, 37]. Therefore, we consider that CCh-induced DG kinase translocation may be produced by phosphorylation of DG kinase by PKC and recovered by serine/threonine phosphatase.

In this report, we conclude that DG kinase translocation is maintained by CCh. The initial transient increase in intracellular calcium, probably released from the intracellular store, is utilized for the translocation of DG kinase from the cytosol to the membrane. At this time, PKC may regulate DG kinase translocation by means of DG kinase phosphorylation, which is supported by the finding that the recovery of DG kinase translocation was suppressed by serine/threonine phosphatase inhibition.

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