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PROTEIN KINASE C IS INVOLVED IN TRANSLOCATION OF DIACYLGLYCEROL KINASE INDUCED BY CARBACHOL IN GUINEA PIG TAENIA COLI

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Abstract—The regulatory mechanisms of diacylglycerol (DG) kinase activity were studied in guinea pig taenia coli. In an octylglycoside mixed micellar assay system, DG kinase activities were distributed in both membrane and cytosolic fractions. Treatment of the tissue with carbachol (CCh) increased the activity in the membrane fraction and decreased the cytosolic fraction without affecting total DG kinase activity. The K_m value of DG kinase in the membrane fraction was unchanged by treatment with CCh, although V_{max} was increased. These findings suggest that DG kinase may be translocated from the cytosol to the membrane by CCh-stimulation. Increase in DG content by treatment of tissue with a cell-permeable species of DG, dioctanoyl-*sn*-glycerol, did not induce DG kinase translocation. Each treatment with protein kinase C (PKC) inhibitor and PKC-desensitization blocked CCh-induced DG kinase translocation; and phorbol ester induced the translocation only in intracellular calcium-accumulated tissues. Considering these results, CCh-induced DG kinase activation appears to involve DG kinase translocation from the cytosol to the membrane in association with both PKC and intracellular calcium concentration rather than cellular DG content.

Key words: 1-stearoyl-2-arachidonyl-*sn*-glycerol; calcium; dioctanoyl-*sn*-glycerol; phosphatidic acid; R59022; signal transduction

DG kinase†, which phosphorylates DG, is responsible for the accumulation of PA and this enzyme activity is present in many types of cells [1, 2]. DG is known to be released from phosphatidylinositol-4,5-bisphosphate and to serve as a second messenger in PKC-mediated signal transduction [3]. The major route for removal of DG is via its phosphorylation to PA, a metabolite which has been reported to activate phosphatidylinositol turnover [4]. DG kinase is therefore likely to prove a key element in the regulation of DG metabolism. We also previously reported the effects of DG kinase inhibition on CCh-induced calcium influx and sustained force development in guinea pig taenia coli [5]. Detailed studies of the regulatory mechanisms of DG kinase activation are needed to elucidate its function in stimulated as well as in non-stimulated cells. Maroney and Macara [6] and Besterman *et al.* [7] reported that DG kinase was translocated from the cytosol to the membrane and that this translocation was induced by increases in cellular DG content, although conclusive evidence was lacking. It is not known to what extent the amount of DG in the membrane determines enzyme activity. Therefore, changes in DG kinase activity and its regulatory mechanisms have not been clearly understood.

We previously demonstrated that the synthetic short-chain DG, diC8, penetrates the cell membrane of guinea pig taenia coli and can be used as an exogenous substrate of DG kinase [8]. Using this method, we showed that CCh activated DG kinase at the tissue level (tissue–DG kinase assay system) in a time- and dose-dependent manner and that activation was blocked by atropine. This receptor-mediated DG kinase activation was regulated not only by cellular substrate concentration but also by other regulatory systems. In the present tissue–DG kinase assay system, DG kinase activation was induced by simultaneous treatment with KCl and PDBu, while no effect was observed with treatment by KCl and PDBu alone. From these results, we suggested that both intracellular calcium concentration and PKC may be related to cellular DG kinase regulation in guinea pig taenia coli.

In the present study, we report that CCh induces the translocation of DG kinase from the cytosol to the membrane, and that this translocation is associated with intracellular calcium concentration and PKC activity in guinea pig taenia coli.

MATERIALS AND METHODS

Materials. Carrier-free and HCl-free [^{32}P]Pi and

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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; Atr, atropine sulphate; CCh, carbachol; DG, diacylglycerol; DG kinase, diacylglycerol kinase; diC8, dioctanoyl-*sn*-glycerol; diC8-PA, dioctanoylphosphatidic acid; PA, phosphatidic acid; PDBu, phorbol-12,13-dibutyrate; [^{32}P]Pi, radioactive inorganic phosphate; PKC, protein kinase C; R59022, 6-[2-(4-[(4-fluorophenyl) phenylmethylene]-1-piperidinyl)ethyl]-7-methyl - 5H - thiazolo[3,2 - α]pyrimidine - 5 - one; octylglycoside, *n*-octyl-β-D-glucopyranoside; PtdSer, L-α-phosphatidyl-L-serine.

[γ-^{32}P]ATP (111.0 GBq/μmol) were purchased from Du Pont-New England Nuclear (Boston, MA, U.S.A.). The DG kinase inhibitor R59022 was obtained from Janssen Life Science Products (Olen, Belgium). Atr, octylglycoside, CCh, PtdSer from bovine brain, leupeptin, pepstatin, 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. (AL, U.S.A.). PDBu was obtained from Seikagaku Co. (Tokyo, Japan). TLC plates, silica gel 60 with a concentrating zone were obtained from Merck Inc. (Darmstadt, Germany). All other chemicals and materials were of reagent grade.

R59022 was dissolved in 10% ethanol solution containing 0.005 M HCl as a 2.5 mM stock solution [9].

Isolation of guinea pig taenia coli. Taenia coli were isolated from guinea pig (200–400 g body weight, either sex) as previously described [10]. Tissue specimens, 10–15 mg wet weight, 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 (pH 7.4) at 37°.

Subcellular fractionation of guinea pig taenia coli. Tissue treated under the various conditions were homogenized with a polytron homogenizer (Brinkman Institute) in an 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 (1000 *g* for 5 min) to remove nuclei. The supernatant was decanted and the pellets washed once with buffer B (sucrose-free buffer A). Then, the combined supernatants were centrifuged again (20,000 *g* for 30 min) and the pellets resuspended in buffer B (mitochondrial fraction). Finally the membranes were collected by centrifugation of the supernatant (100,000 *g* for 60 min) and resuspended in buffer B. The supernatant was used as the cytosolic fraction.

Crude DG kinase preparation and assay of activity by the mixed micellar assay system. Octylglycoside and KCl were added to the resuspended subcellular fractions on ice to final concentrations of 2% and 300 mM, respectively. The mixtures were slowly shaken for 30 min at room temperature and centrifuged at 100,000 *g* for 60 min. Each supernatant was used for the mixed micellar assay system as a crude DG kinase fraction. DG kinase activity was assayed in the presence of diC8 and 18:0/20:4-DG as in previous reports [8]. The extracted enzyme or cytosolic fraction was incubated for 2 min at 37° in the presence of 60 mM MOPS (pH 7.2), 0.86 mM DTT, 18 mM $MgCl_2$, 73 mM octylglycoside, 3.3 mM PtdSer, 0.52 mM [γ-^{32}P]ATP (711 MBq/mol) and various concentrations of diC8 and 18:0/20:4-DG. The reaction was terminated by addition of ice-cold chloroform/methanol/10 M HCl (100:200:1, v/v/v). DG concentrations were expressed as mol% of octylglycoside in micellar form; calculations were based on a critical micellar concentration of 25 mM for octylglycoside. The products, diC8-PA and 18:0/20:4-PA, were extracted and separated as described below.

Assay of DG kinase activity using diC8 in intact tissue. DG kinase activity in intact tissue was determined by measuring the accumulation of [^{32}P]-diC8-PA from diC8 in [^{32}P]Pi- and diC8-prelabelled guinea pig taenia coli. Simultaneously, the endogenous PA level was determined by measuring the accumulation of [^{32}P]PA in each tissue.

In this assay, diC8 was dissolved in chloroform and stored at −10° as a stock solution. Before use, the diC8 stock solution was dried under N_2 gas at room temperature and dissolved in 50% ethanol solution (final 0.03%). The diC8 ethanol solution was added to a Tyrode solution containing 2.7 mg/mL BSA (diC8 solution).

The tissues were first incubated with 2.22 MBq/mL of [^{32}P]Pi in 1 mL of diC8 solution for 30 min at 37°. The tissues were then washed twice with 10 mL Tyrode solution and the reaction started by the addition of 0.8 mL of Tyrode solution containing various compounds. The reaction was terminated by addition of 3 mL ice-cold chloroform/methanol/10 M HCl (100:200:1, v/v/v) and homogenized with 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 and* [^{32}P]-18:0/20:4 *PA and* [^{32}P]*PA.* One millilitre of chloroform and 1 mL 0.1 M HCl solution were added to the reaction mixture. The mixture was vigorously shaken then centrifuged for 20 min at 1000 *g* to allow phase separation [11]. 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 for TLC analysis.

The redissolved chloroform phase was spotted on TLC plates, which were developed using an organic phase of ethylacetate/isooctane/acetic acid/water (7:5:2:1, v/v/v) [12]. In this system, diC8-PA (R_f 0.27) and 18:0/20:4-PA or endogenous-PA (R_f 0.51) were separated from each other and from major phospholipids. The spots corresponding to [^{32}P]diC8-PA and [^{32}P]18:0/20:4-PA as localized by autoradiography (−20°, overnight) were scraped and radioactivities counted by liquid scintillation spectrometry.

Other assays. Total cellular PKC activity was measured according to the method of Valge *et al.* [13]. Protein concentrations were measured by the method of Lowry *et al.* [14] with BSA as a standard.

Data analysis. Data are shown as means ± SE of values obtained in at least four or five separate animals. The significance of differences between the values were assessed by one-way analysis of variance (ANOVA).

RESULTS

Subcellular distribution of DG kinase activity

We first examined the subcellular distribution of DG kinase activity in guinea pig taenia coli. In this assay system, we used two differential species of DGs as substrates: 18:0/20:4-DG as the typical endogenous species of DG [15] and diC8 as a common species of substrate in the tissue–DG kinase assay system. In the guinea pig taenia coli, there was no DG kinase activity in nuclear or mitochondrial fractions, whereas activity was detected in the

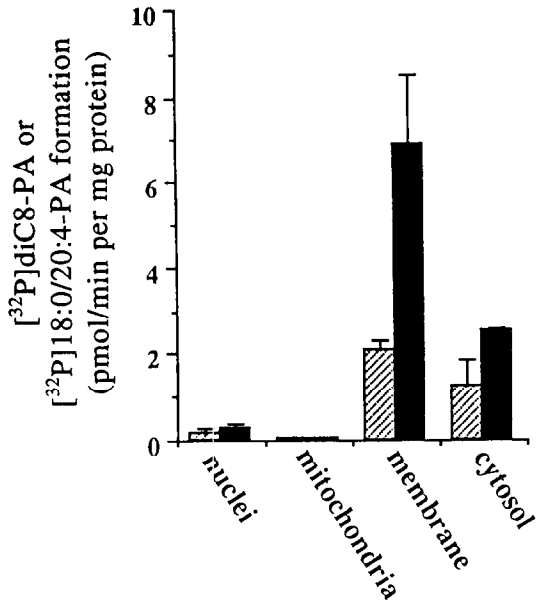

Fig. 1. Subcellular distribution of DG kinase activity in guinea pig taenia coli. Guinea pig taenia coli were homogenized and subcellular fractions were collected. DiC8 and 18:0/20:4-DG were dried under N_2 gas and dispersed in β-octylglycoside micelles. Extracted crude DG kinase from each fraction was incubated with the mixed micelles for 2 min at 37°, and rates of phosphorylation of (▨), diC8, and (■) 18:0/20:4-DG were measured. Each value represents mean ± SE of at least five independent determinations.

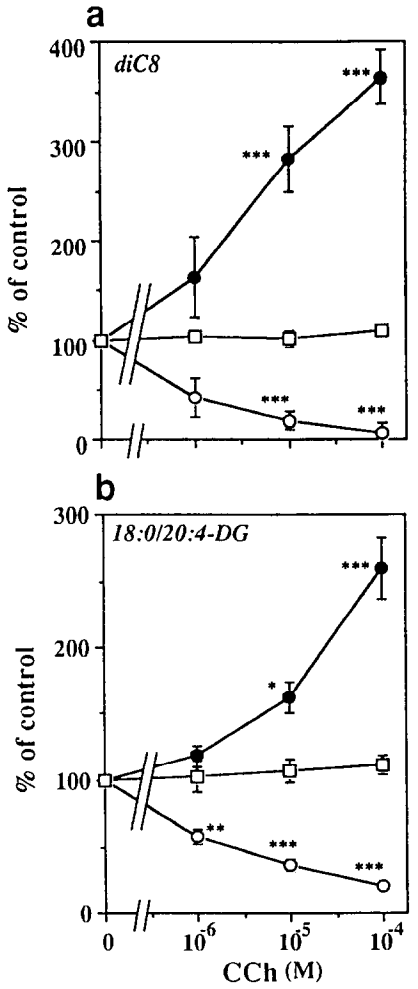

Fig. 2. Effects of CCh on the distribution of DG kinase activity. After guinea pig taenia coli were treated with various concentrations of CCh for 5 min at 37°, the tissue was homogenized and each fraction then collected. Rates of phosphorylation of (a) diC8, and (b) 18:0/20:4-DG in (○) membrane fraction, (●) cytosolic fraction and both fractions (20,000 g supernatant; □) were measured as described for Fig. 1. Results are expressed as percent of non-treated control of each fraction. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ values significantly different from control.

membrane and cytosolic fractions (Fig. 1). The rates of phosphorylation of 18:0/20:4-DG in these fractions were 6.9 ± 1.67 and 2.52 ± 0.08 pmol/min/mg protein, respectively. In both fractions, the rates of diC8 phosphorylation were approximately half those of 18:0/20:4-DG.

In these fractions, the phosphorylation of diC8 and 18:0/20:4-DG were proportional to the incubation time of between 1 and 4 min in the mixed micellar assay system (data not shown). On the basis of the data, subsequent experiments were carried out in an incubation of 2 min.

Pretreatment of the guinea pig taenia coli with 3 μM R59022 as a specific DG kinase inhibitor for 5 min caused 95% inhibition of DG kinase in both fractions while residual activity was abolished when assayed in the presence of 3 μM R59022 in the assay mixture (data not shown).

Subcellular distribution of CCh-induced DG kinase activity

To examine the effects of CCh on DG kinase distribution, fresh tissues were treated with various concentrations of CCh for 5 min at 37°, and each subcellular fraction was then prepared (Fig. 2). Treatment with CCh increased the rates of phosphorylation of diC8 and 18:0/20:4-DG in the membrane fraction. In contrast, the rates of diC8 and 18:0/20:4-DG phosphorylation in the cytosolic fraction were found to decrease. The rate of phosphorylation in 20,000 g supernatant containing both membrane and cytosolic fractions was not changed by CCh treatment.

Then, we examined the effects of CCh on K_m and V_{max} values of DG kinase in the membrane fraction (Fig. 3). Treatment of tissue with 100 μM CCh caused an increase in V_{max} (from control value 3.98 to 13.45 pmol/min/mg protein), but did not affect the K_m value (1.31 mol% for diC8). To examine whether the CCh-induced changes in DG kinase distribution were mediated by muscarinic receptor stimulation, tissues were pretreated with 100 μM CCh for 5 min in the presence of 1 μM Atr (Fig. 4). CCh-induced changes in rates of phosphorylation of

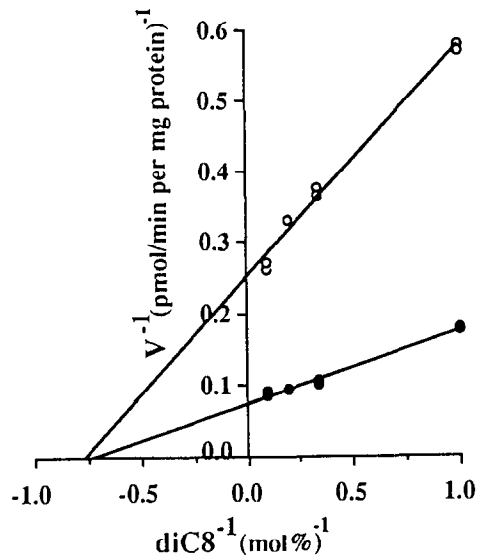

Fig. 3. Lineweaver–Burk plots of DG kinase activity in the membrane fraction. Guinea pig taenia coli were incubated in (○) the presence or (●) the absence of 100 μM CCh for 2 min at 37°, and the membrane fraction prepared. Rates of phosphorylation of various concentrations of diC8 were measured as described for Fig. 1. Each point represents an individual determination.

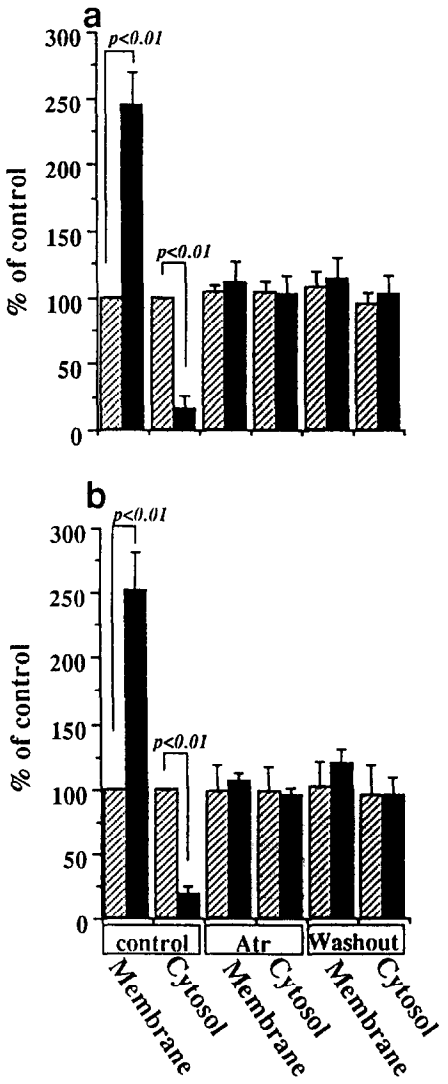

Fig. 4. Effects of Atr and washout on the distribution of DG kinase activity. Fresh tissues were incubated in the absence (control and washout) or presence of Atr (1 μM Atr) for 5 min, and then incubated in (▨) the absence or (■) the presence of 100 μM CCh for 5 min at 37°. Some tissues were then washed four times with Tyrode solution (Washout). Membrane and cytosolic fractions were collected and rates of phosphorylation of (a) diC8, and (b) 18:0/20:4-DG measured. Results are expressed as percent of non-treated control of each fraction.

diC8 and 18:0/20:4-DG in membrane and cytosolic fractions were blocked by this treatment, while control levels were unaffected. When fresh tissues were incubated with 100 μM CCh for 5 min at 37° and then washed four-times with Tyrode solution, CCh-induced changes in the rates of phosphorylation in both fractions returned to control levels. These results indicated that the CCh-induced changes in distribution of DG kinase activity were mediated by muscarinic receptor stimulation and were reversible.

Mechanisms of CCh-induced alterations in DG kinase distribution

We next examined the mechanisms of alterations in DG kinase distribution induced by CCh in guinea pig taenia coli. Some previous reports have suggested that the translocation of DG kinase from the cytosol to the membrane is regulated by DG content [6, 7]. However, we previously reported that submaximal incorporation of diC8 into the tissue occurred in treatment with 100 μM diC8 for 30 min at 37°, but this treatment caused no alterations in cellular DG kinase activity in the tissue-DG kinase assay system [8]. In the present study, we examined the effects of diC8 incorporation into fresh tissues and found no alterations in distribution profiles of DG kinase (Fig. 5). These results indicated that alterations in DG kinase distribution were not induced by increases in cellular DG content.

To clarify the regulatory mechanisms of the change in DG kinase distribution, we examined the effects of PKC inhibition on the CCh-induced change in DG kinase activity in our two assay systems. We used two methods of PKC inactivation: treatment of PKC inhibitor (H-7) and PKC desensitization. Since DG kinase activity is inhibited directly by various reagents at different concentrations, we first examined the direct effect of H-7 on DG kinase activity in a mixed micellar assay system (data not shown). Mixed micelles containing 5 mol% of diC8 or 18:0/20:4-DG were prepared in the presence or absence of H-7, and the rates of phosphorylation of both substrates were then measured. The rates of phosphorylation of diC8 and 18:0/20:4-DG were inhibited by treatment with 3 μM R59022, while the

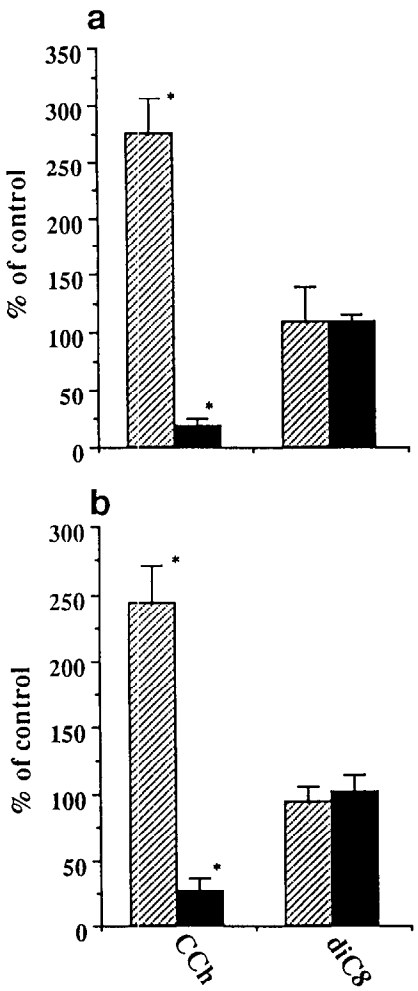

Fig. 5. Effect of diC8 on the distribution of DG kinase activity. Fresh tissues were incubated in the absence (control) or presence of 100 μM CCh for 5 min at 37°, or were treated with 100 μM diC8 for 30 min at 37°. (▨) Membrane, and (■) cytosolic fractions were collected and rates of phosphorylation of (a) diC8, and (b) 18:0/20:4-DG measured. Results are expressed as percent of non-treated control of each fraction. *$P < 0.01$ values significantly different from control.

rate was scarcely reduced by treatment with 10 μM H-7. R59022 was also used as a negative control. Therefore, the concentrations of H-7 were selected in the range of values so as to be effective in inhibiting PKC without causing direct or other non-specific effects.

In the tissue-DG kinase assay system, [^{32}P]Pi- and diC8-prelabelled guinea pig taenia coli were incubated with several concentrations of H-7 for 10 min at 37°, with 100 μM CCh added for the last 5 min of incubation (Fig. 6a and b). In the absence of H-7, 100 μM CCh induced significant increases in accumulation of [^{32}P]diC8-PA (from control value 54.2 ± 8.97 to 207.9 ± 6.63 cpm/mg wet wt tissue) and [^{32}P]PA (from control value 591.2 ± 6.97 to 1371.5 ± 42.14 cpm/mg wet wt tissue). The presence of H-7 reduced the CCh-induced increase in [^{32}P]-diC8-PA and [^{32}P]PA accumulation concentration-dependently without affecting the control levels. Submaximal inhibition was observed with treatment with 10 μM H-7. In the mixed micellar assay system, membrane and cytosolic fractions were also prepared from tissues treated under the same conditions and DG kinase activities were measured, demonstrating that the CCh-induced changes in the rates of diC8 and 18:0/20:4-DG phosphorylation were also inhibited by treatment with 10 μM H-7 without control levels being affected (Fig. 6c and d).

PKC-desensitization was achieved by long-term treatment of phorbol ester. Tissues were pre-incubated with 1 μM PDBu for 24 hr at 4° and [^{32}P]-diC8-PA and [^{32}P]PA accumulation was then measured by the tissue-DG kinase assay system (Fig. 7a and b). In the PKC-desensitized tissues, the CCh-induced increase in [^{32}P]diC8-PA and [^{32}P]PA accumulation was completely abolished. In these tissues, the CCh-induced change in rates of diC8 and 18:0/20:4-DG phosphorylation was also inhibited in the mixed micellar assay system (Fig. 7c and d).

We next used 50 mM KCl and 1 μM PDBu in the mixed micellar assay system (Fig. 8). KCl increased intracellular calcium concentration and PDBu activated PKC under the basal concentration of intracellular calcium in guinea pig taenia coli (data not shown). Single treatments with KCl or PDBu did not affect the rates of diC8 or 18:0/20:4-DG phosphorylation, but simultaneous treatment caused increases in both rates in the membrane fraction and decreases in both rates in the cytosolic fraction. The same results were obtained by a treatment of 10 μM ionomycin rather than 50 mM KCl (data not shown).

Thus, the observed CCh-induced changes in DG kinase distribution were dependent on both intracellular calcium concentration and PKC activity.

DISCUSSION

We have presented evidence of the CCh-induced translocation of DG kinase activity from the cytosol to the membrane in guinea pig taenia coli. Previously, we reported that diC8 and 18:0/20:4-DG were competitively bound to DG kinase extracted from tissue homogenate [8] and that phosphorylations of diC8 and endogenous DG by cellular DG kinase were increased by treating tissue with CCh.

In the present report, we first examined the subcellular distribution of DG kinase activity in guinea pig taenia coli. Approximately 95% of DG kinase activity was distributed in the membrane and cytosolic fractions (Fig. 1). This distribution profile is the same as that reported in rat brain cells [16]. DG kinase activity in both fractions was completely inhibited by treatment with the DG kinase inhibitor R59022 (data not shown). From these results, we considered that this assay system using diC8 was suitable for investigation of DG kinase activity in membrane and cytosolic fractions.

When tissues were incubated with various concentrations of CCh for 30 min at 37°, DG kinase activity in the membrane fraction was increased and that in the cytosolic fraction was decreased in a dose-

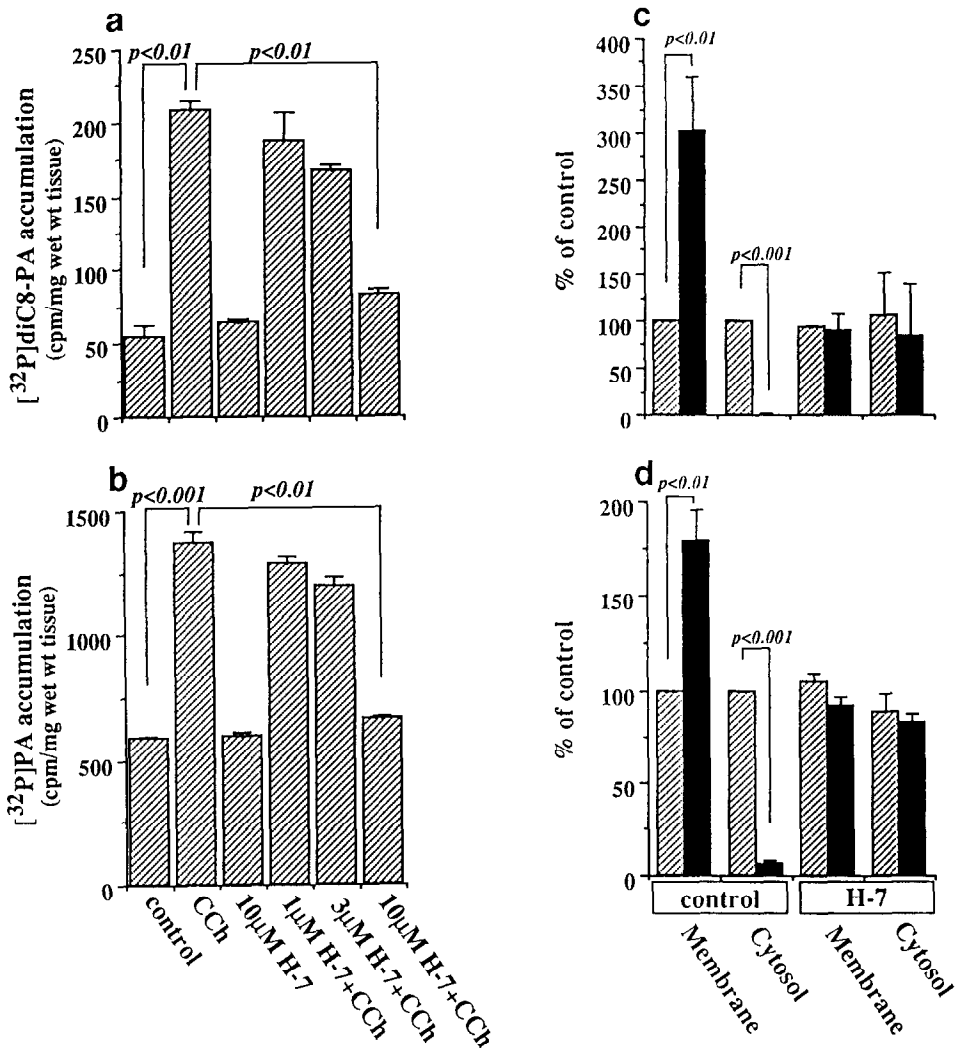

Fig. 6. Effect of H-7 on cellular- and subcellular-DG kinase activity. Fresh tissues were preincubated with 2.22 MBq/mL [^{32}P]Pi and 100 μM diC8 for 30 min at 37°. After being washed, tissues were stimulated in the absence or presence of CCh and/or H-7 at 37°. Tissues were preincubated with various concentrations of H-7 for 5 min, and 100 μM CCh was added for 5 min. (a) [^{32}P]diC8-PA, and (b) [^{32}P]-PA accumulations were quantified as described in the Materials and Methods section. Simultaneously, fresh tissues were preincubated in the presence or absence of 30 μM H-7 for 5 min, and then (▨) unstimulated, or (■) stimulated with 100 μM CCh for 5 min. Membrane and cytosolic fractions were collected and rates of phosphorylation of (c) diC8, and (d) 18:0/20:4-PA measured as described for Fig. 1. Each value represents mean ± SE of at least five independent determinations.

dependent manner (Fig. 2). However, the total DG kinase activity was unchanged by the treatment. The observed changes in DG kinase activity were similar to those of PKC activity which can translocate to membrane [17]. Many studies have investigated the coexpression of DG kinase isozymes in various types of cells [16, 18]. From this point of view, our results also investigated the possibility that one species of DG kinase isozyme in the membrane was activated and another species in the cytosolic fraction was indicated by CCh-treatment. To clarify this point, we measured V_{max} and K_m values of DG kinase in the membrane and cytosolic fractions (Fig. 3). If DG kinase activity in the cytosol of guinea pig taenia coli is due to the same isozyme(s) as that in the membrane, the translocation of DG kinase from the cytosol to the membranes should change V_{max} without affecting K_m values. In the present study, CCh increased V_{max}, but did not affect the K_m values, suggesting that the same DG kinase isozyme is involved in both fractions. We therefore conclude that DG kinase can be translocated from the cytosol to the membrane by the stimulation. In addition, the CCh-induced translocation of DG kinase was mediated by muscarinic receptor stimulation and was redistributed by washout of tissue (Fig. 4).

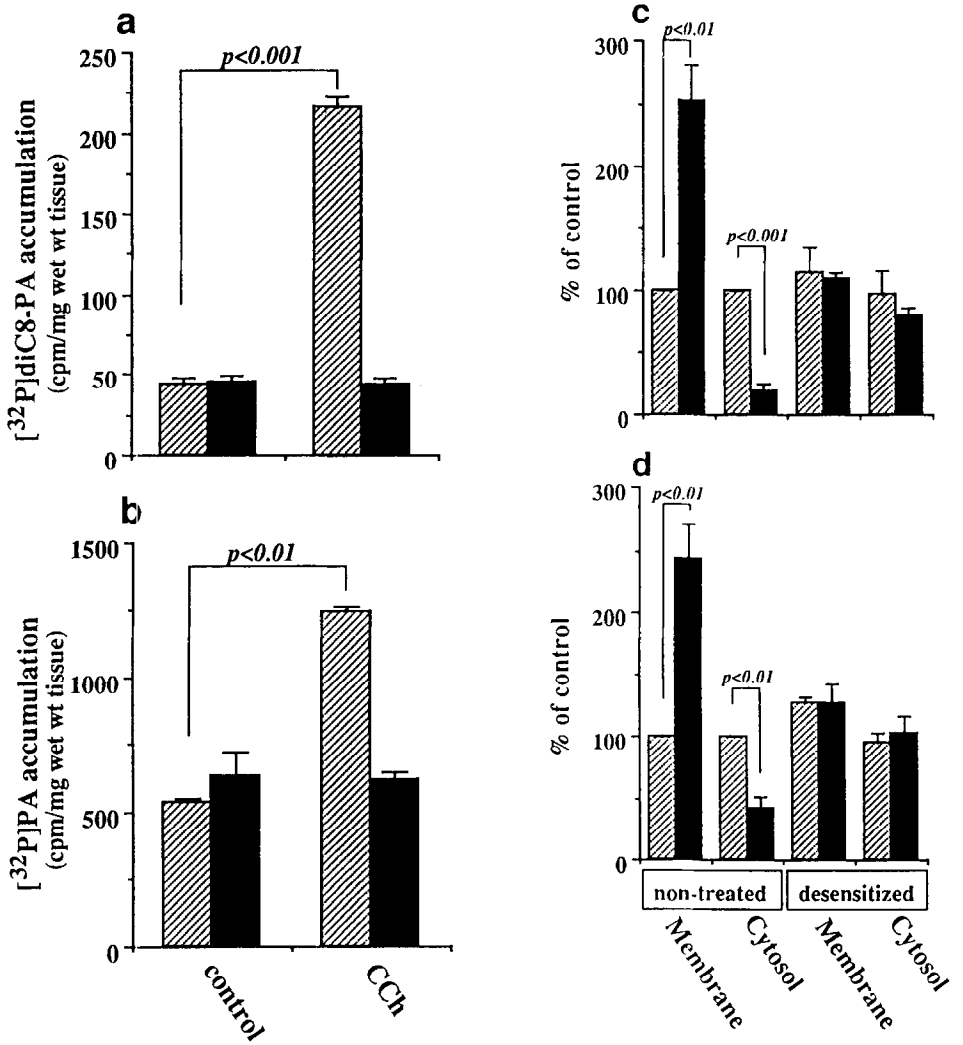

Fig. 7. Effects of PKC desensitization on cellular- and subcellular-DG kinase activity. Fresh tissues were preincubated in (■) the presence, or (▨) the absence of 1 μM PDBu for 24 hr at 4°. The tissues were then incubated with 2.22 MBq/mL [^{32}P]Pi and 100 μM diC8 for 30 min at 37°. After being washed, tissues were incubated in the absence or presence of 100 μM CCh for 5 min at 37°. (a) [^{32}P]diC8-PA, and (b) [^{32}P]PA accumulations were quantified as described in the Materials and Methods section. Each value represents mean ± SE of at least five independent determinations. Preincubated tissues were then simultaneously incubated in (■) the presence, or (▨) the absence of 100 μM CCh for 5 min at 37°. Membrane and cytosolic fractions were collected and rates of phosphorylation of (c) diC8, and (d) 18:0/20:4-PA measured. Results are expressed as percent of non-treated control of each fraction.

Maroney and Maraca reported that DG kinase translocation was regulated by changes in membrane-associated DG content in Swiss 3T3 cells [6]. In contrast, van der Bend *et al.* observed no DG kinase activation by an increase in DG using bacterial phosphatidyl inositol-specific phospholipase C in Jarkat cells [19]. In our previous study, submaximal incorporation of diC8 was observed on treatment with 100 μM diC8 for 30 min at 37°, and this incorporation did not induce cellular DG kinase activation in our tissue-DG kinase assay system [8]. Under the same conditions, submaximal incorporation of diC8 did not cause translocation of DG kinase (Fig. 5); this was consistent with the results obtained in our tissue–DG kinase assay system. From these results, we concluded that CCh-induced DG kinase translocation was not regulated by cellular DG content.

Various regulatory factors of DG kinase have been suggested in many types of cells as described below. Calcium dependency of DG kinase was investigated in an *in vitro* assay system, and Sakane *et al.* reported that the primary structure of the cloned 80 kDa DG kinase cDNA encodes EF-hand motifs typical of calmodulin and other calcium-binding proteins [20]. Moreover, the other regulatory factors of DG kinase activity have also been suggested; Yamada *et al.* showed that cellular DG

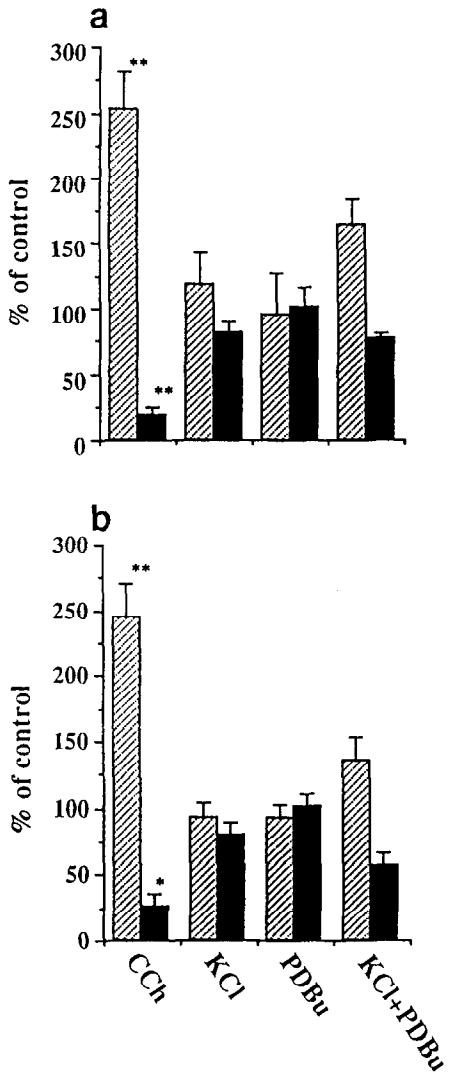

Fig. 8. Effects of KCl and PDBu on the distribution of DG kinase activity. Fresh tissues were incubated in the absence (control) or presence of 100 μM CCh for 5 min at 37°, or were treated with 50 mM KCl and/or 1 μM PDBu for 5 min at 37°. (▨) Membrane, and (■) cytosolic fractions were collected and rates of phosphorylation of (a) diC8, and (b) 18:0/20:4-DG measured. Results are expressed as percent of non-treated control of each fraction. *$P < 0.05$, **$P < 0.01$ values significantly different from control.

kinase was activated directly by treatment with phospholipids such as sphingosine and phosphatidylserine in Jarkat cells [21]. The roles of these agents in cellular DG kinase regulation, however, are not clearly understood. In the present report, we also examined the regulatory factors of DG kinase translocation. When PKC was inhibited by H-7, cellular DG kinase activation induced by CCh was inhibited dose-dependently (Fig. 6a and b) and DG kinase translocation was also inhibited (Fig. 6c and d). Furthermore, PKC desensitization was also caused by inhibition of both cellular DG kinase activation (Fig. 7a and b) and DG kinase translocation (Fig. 7c and d). These inhibitory effects on cellular DG kinase activation may be caused by inhibition of DG kinase translocation. Therefore, we examined the effects of 50 mM KCl and 1 μM PDBu on DG kinase translocation. KCl induced an increase in intracellular calcium concentration and PDBu induced PKC activation. Treatment with both these agents in combination induced DG kinase translocation, while neither treatment singly had any effect (Fig. 8). These findings indicate that CCh-induced DG kinase translocation requires both an increase in intracellular calcium concentration as well as PKC activation in guinea pig taenia coli.

It was not clear whether the intracellular calcium was utilized by DG kinase or PKC, but the calcium dependence of both enzymes has been reported in many types of cells [20, 22]. We infer from this that the intracellular calcium may be utilized by both enzymes. On the other hand, no direct association between DG kinase and PKC has been reported previously in guinea pig taenia coli, but phosphorylation of DG kinase by endogenous protein kinase and PKC-ε was reported in COS-7 cells and pig thymus, respectively [23, 24]. In the light of these reports, DG kinase in guinea pig taenia coli may be regulated by PKC directly.

In guinea pig taenia coli, CCh-induced cellular DG kinase was shown to be involved in DG kinase translocation from the cytosol to the membrane. This translocation may be regulated by both intracellular calcium concentration and PKC activity, but is independent of local substrate concentrations.

REFERENCES

1. Michell RH, Inositol phospholipids and cell surface receptor function. *Biochim Biophys Acta* **415**: 81–147, 1975.
2. Hawthorne JN and Pickard MR, Phospholipids in synaptic function. *J Neurochem* **32**: 5–14, 1979.
3. Nishizuka Y, The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* **308**: 693–698, 1984.
4. Murayama T and Ui M, Phosphatidic acid may stimulate membrane receptors mediating adenylate cyclase inhibition and phospholipid breakdown in 3T3 fibroblasts. *J Biol Chem* **262**: 5522–5529, 1987.
5. Nobe K, Ohata H and Momose K, Effect of diacylglycerol kinase inhibitor, R59022 on cytosolic free calcium level and force development in guinea pig taenia coli. *Res Commun Chem Pathol Pharmacol* **81**: 331–343, 1993.
6. Maroney AC and Macara IG, Phorbol ester-induced translocation of diacylglycerol kinase from the cytosol to the membrane in swiss 3T3 fibroblasts. *J Biol Chem* **264**: 2537–2544, 1989.
7. Besterman JM, Pollenz RS, Booker EL and Cuatrecasas P, Diacylglycerol-induced translocation of diacylglycerol kinase: use of affinity-purified enzyme in a reconstitution system. *Proc Natl Acad Sci USA* **83**: 9378–9382, 1986.
8. Nobe K, Ohata H and Momose K, Activation of diacylglycerol kinase by carbachol in guinea pig taenia coli. *Biochem Pharmacol* **48**: 2005–2014, 1994.
9. de Chaffoy de Courcelles D, Roevens P and Belle HV, R59022, a diacylglycerol kinase inhibitor. *J Biol Chem* **260**: 15762–15770, 1985.
10. Ohata H and Momose K, Phosphatidic acid-induced

contraction in guinea-pig taenia coli. *Res Commun Chem Pathol Pharmacol* **68**: 329–343, 1990.
11. Bligh EG and Dyer WJ, A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**: 911–917, 1959.
12. Yamada K, Sakane F, Imai S and Kanoh H, Sphingosine activates cellular diacylglycerol kinase in intact Jurkat cells, a human T-cell line. *Biochim Biophys Acta* **1169**: 217–224, 1993.
13. Valge VE, Wong JGP, Datlof BM, Sinskey AJ and Rao A, Protein kinase C is required for responses to T cell receptor ligands but not to interleukin-2 in T cells. *Cell* **55**: 101–112, 1988.
14. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
15. Simpson CMF, Itabe H, Reynolds CN, King WC and Glomset JA, Swiss 3T3 cells preferentially incorporate *sn*-2-arachidonoyl-monoacylglycerol into *sn*-1-stearoyl-2-arachidonoyl phosphatidylinositol. *J Biol Chem* **266**: 15902–15909, 1991.
16. Kato M and Takenawa T, Purification and characterization of membrane-bound and cytosolic forms of diacylglycerol kinase from rat brain. *J Biol Chem* **265**: 794–800, 1990.
17. Langlands JM and Diamond J, Translocation of protein kinase C in bovine tracheal smooth muscle strips: the effect of methacholine and isoprenaline. *Eur J Pharmacol* **227**: 131–138, 1992.
18. MacDonald ML, Mack KF, Williams BW, King WC and Glomset JA, A membrane-bound diacylglycerol kinase that selectively phosphorylates arachidonoyl-diacylglycerol. *J Biol Chem* **263**: 1584–1592, 1988.
19. van der Bend RL, de Widt J, Hilkmann H and van Blitterswijk WJ, Diacylglycerol kinase in receptor-stimulated cells converts its substrate in a topologically restricted manner. *J Biol Chem* **269**: 4098–4102, 1994.
20. Sakane F, Yamada K, Kanoh H, Yokoyama C and Tanabe T, Porcine diacylglycerol kinase sequence has zinc finger and E-F hand motifs. *Nature* **334**: 345–348, 1990.
21. Yamada K and Sakane F, The different effects of sphingosine on diacylglycerol kinase isozymes in Jurkat cells, a human T-cell line. *Biochim Biophys Acta* **1169**: 211–216, 1993.
22. Sakane F, Yamada K, Imai S and Kanoh H, Porcine 80-kDa diacylglycerol kinase is a calcium-binding and calcium/phospholipid-dependent enzyme and undergoes calcium dependent translocation. *J Biol Chem* **266**: 7096–7100, 1991.
23. Kanoh H and Ono T, Phosphorylation of pig brain diacylglycerol kinase by endogenous protein kinase. *FEBS Lett* **201**: 97–100, 1986.
24. Schaap D, van der Wal J, van Blitterswijk WJ, van der Bend and Ploegh HL, Diacylglycerol kinase is phosphorylated *in vivo* upon stimulation of the epidermal growth factor receptor and serine/threonine kinases, including protein kinase C-ε. *Biochem J* **289**: 875–881, 1993.