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ACTIVATION OF DIACYLGLYCEROL KINASE BY CARBACHOL IN GUINEA PIG TAENIA COLI

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Abstract—Changes in diacylglycerol kinase (DG kinase) activity in carbachol (CCh)-stimulated guinea pig taenia coli were investigated. In a mixed micellar assay system, added 1,2-dioctanoyl-*sn*-glycerol (diC8) and endogenous DG were competitively bound to common DG kinase isozymes from guinea pig taenia coli and phosphorylated, suggesting that diC8 is useful as a probe of agonist effects on DG kinase activity. In phosphorus-32 ([^{32}P]Pi)- and diC8-prelabeled guinea pig taenia coli, diC8 was phosphorylated by DG kinase to [^{32}P]dioctanoyl-phosphatidic acid ([^{32}P]diC8-PA). CCh increased the accumulation of both [^{32}P]diC8-PA and endogenous [^{32}P]phosphatidic acid ([^{32}P]PA) in a time- and dose-dependent manner (0.1–100 μM CCh). CCh-induced increases in [^{32}P]diC8-PA and [^{32}P]PA were inhibited by 1 μM atropine and 3 μM DG kinase inhibitor (R59022). These findings indicated the activation of DG kinase by muscarinic receptor stimulation in guinea pig taenia coli. Therefore, DG kinase activation may play an important role in CCh-induced PA formation. CCh-induced [^{32}P]diC8-PA and [^{32}P]PA accumulation was dependent on intracellular calcium concentrations. However, a KCl-induced increase in intracellular calcium, without receptor stimulation, was ineffective. Moreover, treatment with phorbol ester also increased accumulation of both PA species in KCl-treated tissues. These findings suggest that muscarinic receptor mediated activation of DG kinase may require both an increase in intracellular calcium and PKC activation in guinea pig taenia coli.

Key words: L-α-1,2-dioctanoyl-*sn*-glycerol; 1-stearoyl-2-arachidonyl-*sn*-glycerol; phosphatidic acid; R59022; calcium; protein kinase C

DG kinase† catalyses the phosphorylation of DG. This enzyme is considered to play a physiological role in the metabolism of the intracellular second messenger, DG, and the formation of PA. DG is widely known as a PKC activator and as such plays a role in signal transduction [1]. On the other hand, different roles of PA have been reported in many types of cells [2, 3]. Its possible role in smooth muscle contraction has also been proposed and this is supported by PA's ability to induce the contraction of smooth muscle cells by increasing intracellular calcium accumulation [4]. We reported previously that the addition of PA induces intracellular calcium accumulation [5] and sustained contraction [6] in guinea pig taenia coli. Whether PA acts as a calcium channel opener [7] or a calcium ionophore [4] remains unknown, but it may play an important physiological role [8]. In cellular PA generation, three major synthesis pathways may be considered: (1) phospholipase C (PLC)-catalysed hydrolysis of PtdIns in conjunction with DG kinase; (2) *de novo* synthesis of lysophosphatidic acid [9, 10]; and/or (3) hydrolysis of PtdCho by phospholipase D (PLD) [11–13]. We have reported that carbahcol (CCh) stimulation increased total PA content in guinea pig taenia coli, but that total DG content remained unchanged [5]. Therefore, we suggested that DG kinase was activated by receptor-coupled CCh stimulation. DG kinase occurs widely in various types of cells [14–18], while DG kinase activation induced by receptor stimulation has only been detected in cell-free systems [19]. However, activation of DG kinase by receptor-mediated stimulation has not been reported at the tissue level.

DG kinases were initially purified from the cytosolic fractions of pig brain [20] and liver extracts [21], and had molecular masses of approximately 80 and 120 kDa, respectively. Subsequently, it was suggested that other isozymes existed in other cell types [22–25]. Sakane *et al.* have investigated cDNA cloned from 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 [26], in addition to zinc finger-like sequences similar to those occurring in PKC. In the purified DG kinase assay system, therefore, calcium and phospholipids such as phosphatidylserine (PS) were used as direct activators. The activation of DG kinase, however, is not clearly understood in agonist-stimulated cellular signaling.

We report here that DG kinase is activated by the

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† Abbreviations: 18:0/20:4-DG, 1-stearoyl-2-arachidonyl-*sn*-glycerol; Atr, atropine sulfate; CCh, carbachol; DG, diacylglycerol; DG kinase, diacylglycerol kinase; diC8, dioctanoyl-*sn*-glycerol; diC8-PA, dioctanoylphosphatidic acid; PA, phosphatidic acid; [^{32}P]Pi, radioactive inorganic phosphate; PtdIns, phosphatidylinositol; PtdCho, phosphatidylcholine; R59022, 6-[2-(4-[(4-fluorophenyl)-phenylmethylene]-1-piperidinyl)ethyl]-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidine-5-one; PKC, protein kinase C; PDBu, phorbol-12,13-dibutyrate.

application of CCh, demonstrating the existence of receptor-coupled DG kinase in guinea pig taenia coli.

MATERIALS AND METHODS

Materials. Carrier-free 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.). A DG kinase inhibitor, R59022, was obtained from Janssen Life Science Products (Olen, Belgium). Atr, *n*-octyl-β-D-glucopyranoside (octylglycoside), CCh, L-α-phosphatidyl-L-serine from bovine brain (PtdSer), leupeptin, pepstatin, soybean trypsin inhibitor (Type 1-S) and calf intestinal phosphatase were purchased from the 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). HPTLC 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 [27].

Isolation of guinea pig taenia coli. Taenia coli were isolated from guinea pig (200–400 g body weight, either sex) as described previously [6]. 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°.

Crude DG kinase preparation and assay of activity by the mixed micellar method. Fresh tissues were homogenized with a polytron homogenizer (Brinkman Institute) in 3 mL of ice-cold solution consisting of 20 mM MOPS (pH 7.2), 250 mM sucrose, 1 mM dithiothreitol (DTT), 1 mM EGTA, 1 μg/mL pepstatin, 1 μg/mL leupeptin and 50 μg/mL soybean trypsin inhibitor. Octylglycoside and KCl were added to the homogenate on ice to final concentrations of 2% and 300 mM, respectively. The mixture was slowly shaken for 30 min and then centrifuged for 60 min at 100,000 *g*.

DG kinase activity was assayed in the presence of diC8 and/or 18:0/20:4-DG according to a modification of the procedure of MacDonald *et al.* [28]. The extracted enzyme 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/or 18:0/20:4-DG. The reaction was terminated by the addition of ice cold chloroform/methanol/10 M HCl (100:200:1, by vol.). Lipid concentration referred to the molar fraction of lipid in the micellar phase of octylglycoside; the 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 the diC8 in intact guinea pig taenia coli. DG kinase activity was determined by measuring the accumulation of [^{32}P]-diC8-PA from diC8 in [^{32}P]Pi- and diC8-prelabeled guinea pig taenia coli (tissue-DG kinase assay system). 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 2220 GBq/mL of [^{32}P]Pi in 1 mL diC8 solution at 37° for 30 min. The tissues were then washed twice with 10 mL Tyrode solution and the reaction started by the addition of 0.8 ml Tyrode solution containing various compounds. The reaction was terminated by the addition of 3 mL ice cold chloroform/methanol/10 M HCl (100:200:1, by vol.). The tissues were homogenized with a glass homogenizer in ice-cold water and [^{32}P]diC8-PA and [^{32}P]PA were extracted as described below.

Extraction and analysis of [^{32}P]*diC8-PA and* [^{32}P]-*PA.* One millilitre of chloroform and 1 mL 0.1 M HCl solution were added to the homogenate and the mixture was vigorously shaken then centrifuged for 20 min at 1000 *g* to allow phase separation [29]. The lower phase was carefully removed and dried under N_2 gas at room temperature. The residue was then redissolved in 40 μL chloroform for TLC analysis.

The redissolved chloroform phase was spotted on HPTLC plates which were developed using the organic phase of ethylacetate/isooctane/acetic acid/water (18:10:6:2, by vol.) [30]. In this system, diC8-PA (R_f 0.27) and PA (R_f 0.51) were separated from each other and from major phospholipids. The spots corresponding to the [^{32}P]diC8-PA and [^{32}P]-PA localized by autoradiography (−20°, over night) were scraped and radioactivities counted by liquid scintillation spectrometry.

To check the accuracy of this system, the separated [^{32}P]diC8-PA and [^{32}P]PA zones on the TLC plate were scraped and extracted with chloroform. These chloroform solutions were analysed according to the method described previously [4]. Under these conditions, [^{32}P]diC8-PA and [^{32}P]PA were displayed as described single bands. Therefore, no other phospholipids overlapped the [^{32}P]diC8-PA and [^{32}P]-PA zones.

Identification of diC8 metabolites. The diC8 metabolites were measured by a modification of the procedure of Bishop and Bell [31]. Tissues were incubated in diC8 solution and diC8 metabolites such as PA, PtdIns and PtdCho in lipid fraction were then extracted as described above. Each metabolite was separated by TLC in chloroform/methanol/water/acetic acid (25:15:4:2 by vol.) using PA, PtdIns and PtdCho as standards. Free-diC8 in DG fraction was quantified as described previously [4]. Separated bands on TLC plates were scraped and extracted by chloroform. Each diC8 metabolite in PA, PtdIns and PtdCho fractions was converted to diC8 using calf intestinal phosphatase. These chloroform solutions were taken to dryness under

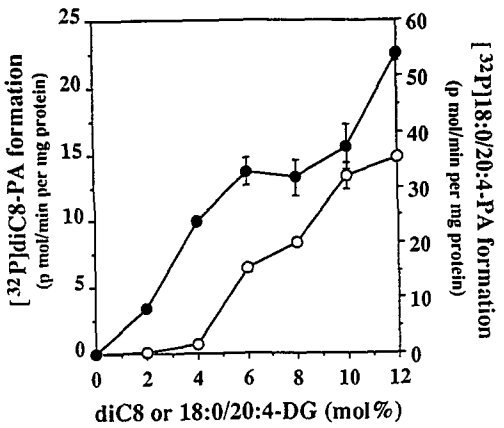

Fig. 1. Phosphorylation of diC8 and 18:0/20:4-DG by extracted crude DG kinase from guinea pig taenia coli. DiC8 or 18:0/20:4-DG was dried under N_2-gas and dispersed in octylglycoside micelles. Extracted crude DG kinase was incubated with the mixed micelles for 2 min at 37° and formation of [^{32}P]diC8-PA (○) and [^{32}P]18:0/20:4-PA (●) were quantified as described in Materials and Methods. Each value represents the mean ± SE of at least five independent determinations.

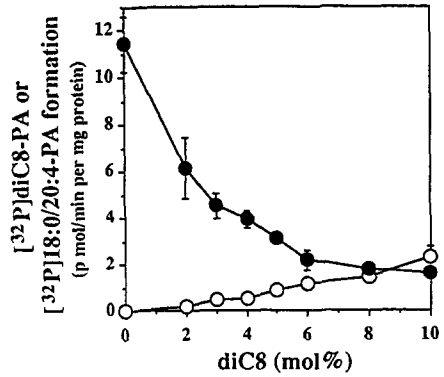

Fig. 2. Inhibitory effect of increase in [^{32}P]diC8-PA formation on [^{32}P]18:0/20:4-PA. Mixtures of 18:0/20:4-DG (final concentration; 2 mol%) and diC8 (final concentrations; 0–10 mol%) were dried under N_2 gas and dispersed in octylglycoside micelles. Extracted crude DG kinase was incubated with the mixed micelles for 2 min at 37° and formation of [^{32}P]diC8-PA (○) and [^{32}P]18:0/20:4-PA (●) was quantified as described in Materials and Methods. Each value represents the mean ± SE of at least five independent determinations.

N_2 and resuspended in 0.5 mL phosphatase buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine). Phosphatase (500 U) was added and the reaction allowed to proceed for 60 min at room temperature. Reconverted diC8 from each fraction was quantified as described previously [4].

Measurements of change in intracellular calcium concentration and force development. Changes in intracellular calcium concentration and force development were simultaneously measured by the fura-2 method as described previously [4].

Other assay. Protein concentrations were measured by the method of Lowry *et al.* [32] with serum albumin as 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 values was assessed by the one-way analysis of variance (ANOVA).

RESULTS

Phosphorylation of diC8 and 18:0/20:4-DG in mixed micelles by crude DG kinase isolated from guinea pig taenia coli

We first considered the possibility that the unusual species of DG, diC8, can be phosphorylated by DG kinase as can the typical endogenous species of DG from PtsIns(4, 5)P_2, 18:0/20:4-DG [33]. The utilization of this diacylglycerol as substrate was compared to that of diC8 by measuring rates of phosphorylation of these compounds in a mixed micellar assay system in which octylglycoside was used to disperse the lipids. In studies of *E. coli* and Swiss 3T3 DG kinase, Walsh and Bell [34] and MacDonald *et al.* [35] demonstrated that the utilization of lipid substrates in mixed micelles is

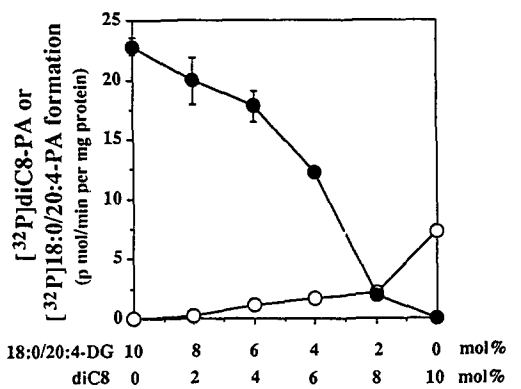

Fig. 3. Competition between diC8 and 18:0/20:4-DG for phosphorylation by crude DG kinase extracted from guinea pig taenia coli. Mixtures of diC8 and 18:0/20:4-DG were dried under N_2 gas and dispersed in octylglycoside micelles. The total amount of the two species of diacylglycerols was kept constant at 10 mol% during incubation. Diacylglycerol mixtures in each combination were incubated as described for Fig. 2. [^{32}P]diC8-PA (○) and [^{32}P]18:0/20:4-PA (●) formations were quantified as described in Materials and Methods. Each value represents the mean ± SE of at least five independent determinations.

independent of the total concentration of lipid in the detergent micelles. We obtained similar results in assays of DG kinase activity extracted from guinea pig taenia coli (data not shown). The phosphorylation of DG species of different molecular masses were compared using an equal molar (10 mol%) fraction calculated as described in Materials and Methods. Under these conditions, the rates of phosphorylation

of both substrates were proportional to the amount of cell protein in the assay (data not shown). Both diC8 and 18:0/20:4-DG were phosphorylated in a mixed micellar assay system in a concentration-dependent manner; the phosphorylation rates of diC8 and 18:0/20:4-DG at 12 mol% were 14.8 ± 0.8 and 53.9 ± 1.1 pmol/min per mg protein, respectively (Fig. 1). To determine whether diC8 and 18:0/20:4-DG can be phosphorylated by a common DG kinase isozyme, we prepared mixed micelles in which the 18:0/20:4-DG was kept constant at 2 mol% and to which various concentrations of diC8 were added (Fig. 2). The rate of diC8 phosphorylation increased dose dependently, but that of 18:0/20:4-DG phosphorylation was markedly reduced by addition of diC8. This inhibitory effect was dependent on the diC8 concentration, nearly maximal inhibition being obtained with 10 mol% diC8. To determine whether the preferential phosphorylation of 18:0/20:4-DG could account for competition with diC8 for phosphorylation, mixtures were prepared in which the proportions of the two substrates were varied while total diacylglycerol concentration was kept constant at 10 mol% (Fig. 3). In this experiment, as a small amount of diC8 was substituted for 18:0/20:4-DG in all 18:0/20:4-DG micelles, the rate of phosphorylation of 18:0/20:4-DG decreased sharply while that of diC8 phosphorylation increased. Equal rates of phosphorylation for both species occurred in a mixture 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 the mixture containing equal amounts (5 mol%) of each species, the rate of phosphorylation of 18:0/20:4-DG was more than 4-fold that of diC8.

Accumulation of [^{32}P]*diC8-PA in* [^{32}P]*Pi and diC8-prelabeled guinea pig taenia coli*

To investigate how DG kinase participates in [^{32}P]-diC8-PA accumulation, we first developed a DG kinase assay system using guinea pig taenia coli (tissue-DG kinase assay system). When tissues were incubated with diC8 at 37° for 30 min, the distribution of diC8 was 57.1 ± 5.8% in free DG, 28.1 ± 4.1% in PA, 7.2 ± 0.9% in PtdIns and 3.2 ± 0.1% in PtdCho. Prelabeling for 3 hr reduced the distribution of diC8 in PA to <10% but increased that of diC8 in PtdIns and PtdCho fractions to ~20% in both fractions (Fig. 4a). In addition, diC8 was incorporated dose-dependently into the guinea pig taenia coli and converted to [^{32}P]diC8-PA (Fig. 4b). This accumulation was enhanced by 100 μM CCh, with a maximal increase being detected in tissue prelabeled with 100 μM diC8 (from control value of 58.5 ± 15.8 to 182.9 ± 19.0 cpm/mg wet weight; $P < 0.05$). In the experiments conducted thereafter, we chose prelabeling conditions of 100 μM diC8 at 37° for 30 min. Under these conditions, with the simultaneous addition of 2220 GBq/mL [^{32}P]Pi to diC8 solution, we found nearly maximal levels of [^{32}P]Pi incorporation into the total phospholipid and PA fractions. Incorporation of diC8 and [^{32}P]Pi did not affect the total mass of PA (data not shown).

Time- and dose-dependency of [^{32}P]*diC8-PA and* [^{32}P]*PA accumulation*

We investigated the time courses of accumulation

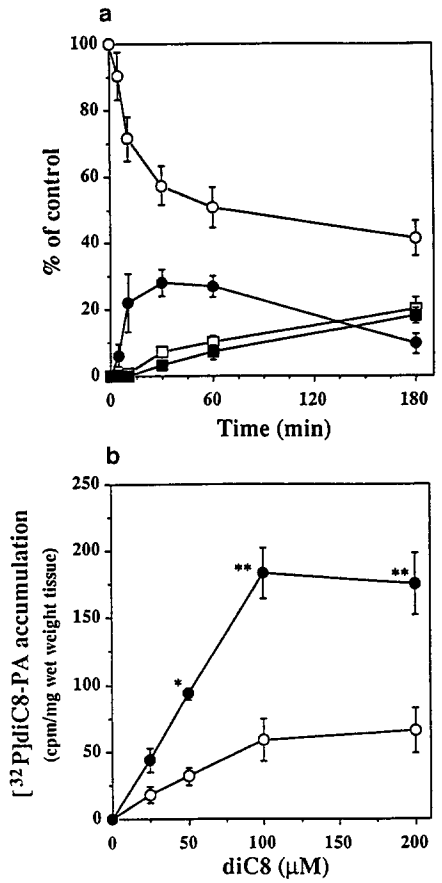

Fig. 4. Time- and dose-dependency of diC8 incorporation. Incorporation of diC8 to some lipid fractions was determined (a). DiC8 (100 μM final) was added to guinea pig taenia coli at 0 min. At the indicated times, diC8 metabolites in PA (●), PtdIns (□), PtdCho (■) and free-DG (○) fractions were extracted and analysed as described in Materials and Methods. The results are presented as per cent of total incorporated diC8. Dose-dependency of diC8 incorporation to diC8-PA fraction was determined (b). Guinea pig taenia coli were preincubated with 2220 GBq/mL [^{32}P]Pi in various concentrations of diC8 solutions at 37° for 30 min. After being washed, the tissues were incubated for 5 min in the absence (○) or presence (●) of 100 μM CCh. [^{32}P]diC8-PA was quantified as described in Materials and Methods. Each value represents the mean ± SE of at least four independent determinations. *$P < 0.05$, **$P < 0.01$, values significantly different from control.

of both [^{32}P]diC8-PA and [^{32}P]PA in the presence and absence of 100 μM CCh in [^{32}P]Pi-and diC8-prelabeled guinea pig taenia coli (Fig. 5). No increase in accumulation of [^{32}P]diC8-PA was detected in the absence of CCh (control); indeed, a very small decrease was observed in basal accumulation of [^{32}P]diC8-PA after 10 min incubation in Tyrode solution. In the presence of 100 μM CCh, however, a significant increase in accumulation of [^{32}P]-diC8-PA (from basal value 61.9 ± 11.3 to 181.8 ± 23.2 cpm/mg wet weight tissue) was detected

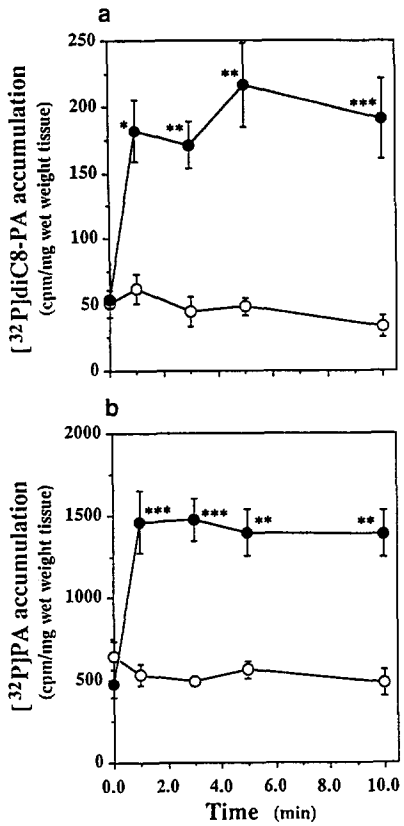

Fig. 5. Time course of CCh induced [^{32}P]diC8-PA and [^{32}P]-PA accumulation in guinea pig taenia coli. [^{32}P]Pi- and diC8-prelabeled guinea pig taenia coli were incubated in the absence (○) or presence (●) of 100 μM CCh at 37°. [^{32}P]diC8-PA (a) and [^{32}P]PA (b) accumulation was quantified as described in Materials and Methods. Each value represents the mean ± SE of at least four independent determinations. *P < 0.05, **P < 0.01, ***P < 0.001 values significantly different from control.

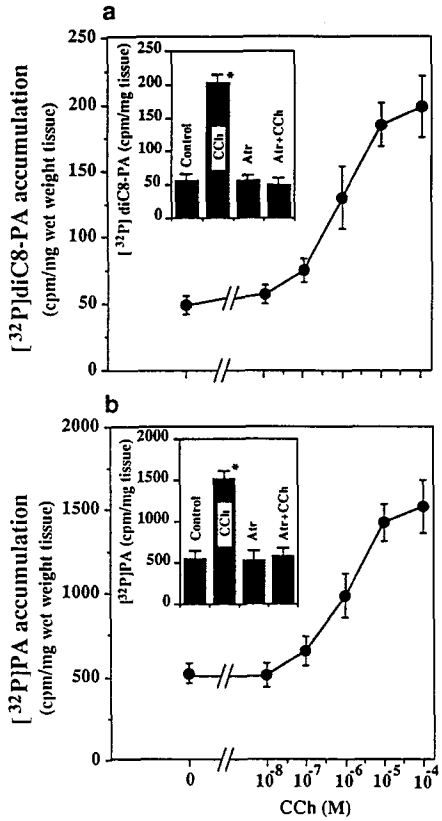

Fig. 6. Dose–response curve for CCh-induced muscarinic receptor-mediated [^{32}P]diC8-PA and [^{32}P]PA accumulations in guinea pig taenia coli. [^{32}P]Pi and diC8-prelabeled guinea pig taenia coli were incubated with various concentrations of CCh for 5 min. [^{32}P]Pi- and diC8-prelabeled guinea pig taenia coli were stimulated in the absence or presence of CCh and/or Atr at 37°. Tissues were incubated with 100 μM CCh and 1 μM Atr individually for 5 min and 10 min, respectively. To determine the effects of Atr on CCh stimulation, the guinea pig taenia coli were preincubated with 1 μM Atr for 5 min, and 100 μM CCh was added for 5 min (inset). [^{32}P]diC8-PA (a) and [^{32}P]PA (b) accumulation was quantified as described in Materials and Methods. Each value represents the mean ± SE of at least five independent determinations. *P < 0.001 values significantly different from control.

after 1 min incubation. This reached a maximal 4-fold increase over basal values (at 267.7 ± 31.0 cpm/mg wet weight tissue) after 5 min of stimulation and was sustained over the remainder of the 30 min incubation period. Similarly, CCh-induced [^{32}P]PA accumulation synthesized from endogenous DG increased rapidly and reached maximal levels 3-fold greater than the control level (533.4 ± 63.4 cpm/mg wet weight tissue). The time course of CCh-induced change in [^{32}P]diC8-PA accumulation corresponded well to the changes in [^{32}P]PA levels. CCh-induced accumulation of [^{32}P]diC8-PA and [^{32}P]PA increased with the increase in concentration (Fig. 6). Incubation for 5 min with increasing CCh concentrations caused maximal accumulation of both [^{32}P]diC8-PA and [^{32}P]PA, i.e. an approximately 4-fold increase over the control value. The calculated EC_{50} of CCh for accumulation of [^{32}P]diC8-PA and [^{32}P]PA were 0.78 and 1.02 μM, respectively. The increase in accumulation of [^{32}P]diC8-PA and [^{32}P]PA induced by 100 μM CCh was almost completely inhibited by 1 μM atropine, a specific antagonist of the muscarinic receptor (Fig. 6 inset), indicating that CCh-induced activation of DG kinase is mediated through the muscarinic receptor stimulation.

Effects of R59022 on CCh-induced [^{32}P]diC8-PA and [^{32}P]PA accumulation

Incubation with 3 μM R59022 for 10 min did not inhibit the control values of the accumulation of [^{32}P]diC8-PA and [^{32}P]PA (Fig. 7). Pretreatment with 3 μM R59022 inhibited the accumulation of both [^{32}P]diC8-PA and [^{32}P]PA induced by 100 μM CCh. When the accumulation of [^{32}P]diC8-PA and [^{32}P]PA induced by CCh was taken as 100%, R59022 inhibited accumulation by 95.1 and 55.2%, respectively, and thereby had a selective effect on [^{32}P]diC8-PA accumulation.

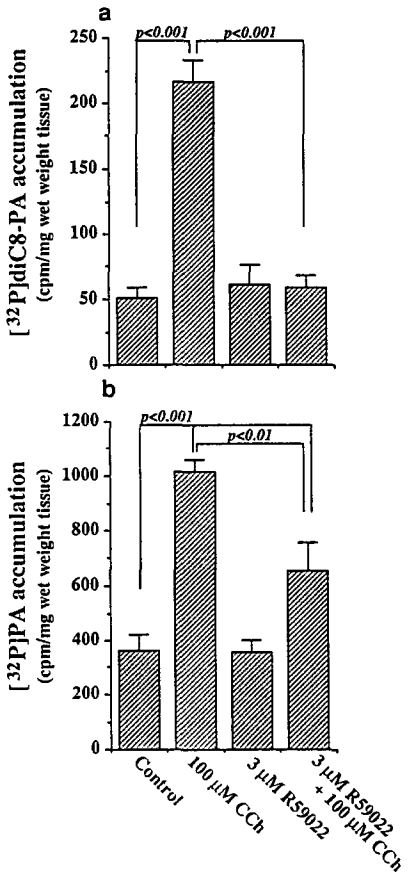

Fig. 7. Effects of R59022 on CCh-induced [^{32}P]diC8-PA and [^{32}P]PA accumulation in guinea pig taenia coli. [^{32}P]-Pi- and diC8-prelabeled guinea pig taenia coli were stimulated in the absence or presence of CCh and/or R59022 at 37°. Tissues were preliminarily incubated with 100 μM CCh and 3 μM R59022 for 5 and 10 min, respectively. To determine the effects of R59022 on CCh stimulation, the guinea pig taenia coli were preincubated with 3 μM R59022 for 5 min, and 100 μM CCh was added for 5 min. [^{32}P]diC8-PA (a) and [^{32}P]PA (b) accumulation was quantified as described in Materials and Methods. Each value represents the mean ± SE of at least five independent determinations.

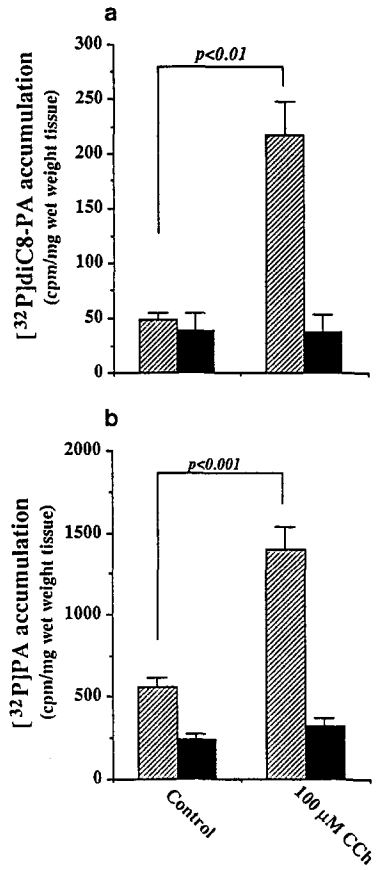

Fig. 8. Effects of intracellular calcium on CCh-induced [^{32}P]diC8-PA and [^{32}P]PA accumulation in guinea pig taenia coli. [^{32}P]Pi and diC8-prelabeled guinea pig taenia coli were stimulated in the absence or presence of CCh at 37° for 5 min in Tyrode solution (▨). Ca^{2+}-free Tyrode solution was changed before 5 min of stimulation (■). [^{32}P]diC8-PA (a) and [^{32}P]PA (b) accumulation was quantified as described in Materials and Methods. Each value represents the mean ± SE of at least five independent determinations.

Effects of intracellular calcium concentration on [^{32}P]diC8-PA and [^{32}P]PA accumulation

Many reports have investigated the presence of calcium as a prerequisite for the activation of DG kinase in cell-free systems. However, the effects of intracellular calcium on DG kinase activity in intact tissues are not clearly understood. To investigate the effects of intracellular calcium concentration on CCh-induced DG kinase activation, we used calcium-free Tyrode solution containing 5 mM EGTA (Ca^{2+}-free Tyrode solution). Ca^{2+}-free Tyrode solution was changed before 5 min of CCh application. In fura-2-loaded guinea pig taenia coli incubated under the same conditions, changes in intracellular calcium concentration and force development by 100 μM CCh treatment were completely abolished without control levels being affected (data not shown). Accumulation of [^{32}P]diC8-PA and [^{32}P]PA in the absence of CCh was not significantly different from the control levels of both molecular species in Tyrode solution, and 100 μM CCh did not affect accumulation of either molecular species in Ca^{2+}-free Tyrode solution (Fig. 8).

To increase the intracellular calcium level without receptor-mediated stimulation, we next used 50 mM KCl. Fura-2-loaded tissues were depolarized with 50 mM KCl, which increased intracellular calcium concentration (data not shown). Under these conditions, [^{32}P]Pi- and diC8-prelabeled guinea pig taenia coli were incubated with 50 mM KCl at 37° for 5 min, and the result was that KCl did not increase [^{32}P]diC8-PA and [^{32}P]PA accumulation (Fig. 9).

Effects of PKC activity on [^{32}P]diC8-PA and [^{32}P]-PA accumulation

The possible role of PKC in the regulation of DG

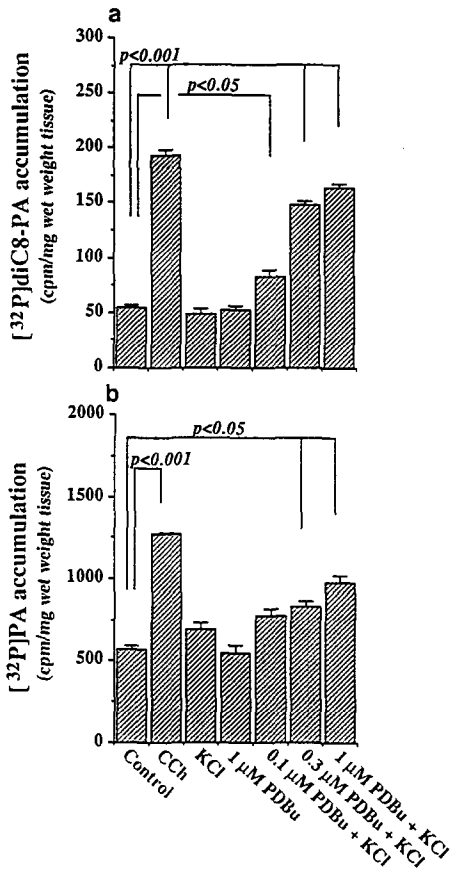

Fig. 9. PDBu- and KCl-induced [32P]diC8-PA and [32P]PA accumulation. [32P]Pi- and diC8-prelabeled guinea pig taenia coli were incubated in the presence or absence of KCl and/or various concentrations of PDBu at 37° for 5 min. CCh of 100 μM was used as a positive control. [32P]-diC8-PA (a) and [32P]PA (b) accumulation was quantified as described in Materials and Methods. Each value represents the mean ± SE of at least five independent determinations.

kinase activity was investigated by testing the effect of PDBu as a specific PKC activator. PDBu concentration was selected in the range of values reported to be maximally effective in activating PKC without causing other non-specific effects. In [32P]-Pi- and diC8-prelabeled guinea pig taenia coli, accumulation of [32P]diC8-PA and [32P]PA were induced by 100 μM CCh but not by 50 mM KCl as described above. Single treatment with 1 μM PDBu at 37° for 5 min did not affect [32P]diC8-PA and [32P]PA accumulation (Fig. 9). To investigate the effects of increases in intracellular calcium concentration, we performed this experiment in the presence of 50 mM KCl. Exposure of [32P]Pi- and diC8-prelabeled guinea pig taenia coli to various concentrations of PDBu in the presence of 50 mM KCl induced [32P]diC8-PA and [32P]PA accumulation in a concentration-dependent manner. When [32P]-diC8-PA and [32P]PA accumulation induced by 100 μM CCh was taken as 100%, that induced by 1 μM PDBu in the presence of 50 mM KCl was 79.1 ± 2.3 and 58.5 ± 5.6%, respectively.

DISCUSSION

Many previous studies have shown that PtdIns-turnover plays an important role in signal transduction of calcium-mobilizing agents [36, 37]. According to the current hypothesis, the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) to inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and DG triggers signaling to the cell, and products then mediate some intracellular hormonal actions [1, 38]. Moreover, PA is increased in response to hormonal stimulation and is involved in signal transduction. The role of DG kinase in PA synthesis has been investigated using membrane fragments [39] and purified enzymes [40, 24], by determining incorporation of radioactivity from organic phosphate into PA, while cells and tissues have rarely been examined. Phosphorylation of incorporated diC8 into dispersed rabbit aorta cells [19] and Jurkat cells [30] has been reported, but stimulation of phosphorylation by receptor agonists has not yet been examined.

In the present study, we demonstrated that CCh stimulated the phosphorylation of exogenously administered diC8 which penetrate the cell membranes in isolated guinea pig taenia coli, suggesting that DG kinase was activated by an agonist. However, previous reports have shown that some DG kinase isozymes described to date coexist in a single type of cells [28] and have different substrate selectivities. Although artificial short chain DGs such as diC8 are commonly phosphorylated by many types of DG kinase isozymes [30], the number of isozymes and their substrate selectivity are unknown in guinea pig taenia coli. Moreover, using a cell-free DG kinase assay system, Yamada and Sakane [41] detected an 18:0/20:4-DG-specific DG kinase in Jurkat cells by the octylglycoside mixed micellar assay system but not by the deoxycholate suspension method. To identify the appropriate DG kinase assay system, we investigated DG kinase activity in guinea pig taenia coli using combinations of different DGs as substrates and two different assay methods: the octylglycoside mixed micellar assay system and the deoxycholate suspension method. In the latter, the rates of phosphorylation of diC8 and 18:0/20:4-DG were not reproducibly time or dose dependent (data not shown). On the other hand, in the octylglycoside mixed micellar assay system, the rates of diC8 and 18:0/20:4-DG phosphorylation were both time and concentration dependent (Fig. 1), and linear with respect to DG kinase concentration. The rate of 18:0/20:4-DG phosphorylation was similar to that of baboon muscle DG kinase activity [42]. Therefore, we chose the octylglycoside mixed micellar assay system to investigate the properties of DG kinases extracted from guinea pig taenia coli. To determine whether diC8 could be phosphorylated by the same DG kinase isozymes as those which phosphorylate 18:0/20:4-DG, we next incubated extracted crude DG kinase with mixtures of diC8 and 18:0/20:4-DG (Fig. 2). The results indicate that diC8 and 18:0/20:4-DG bound competitively

to common DG kinase isozymes. Measurement of the competition between diC8 and 18:0/20:4-DG for phosphorylation showed that the rate of phosphorylation of 18:0/20:4-DG was more than 4-fold higher than that of diC8 (Fig. 3). Some reports established that DG kinase activity was detected in soluble and membrane associated fractions [21, 28]. We also demonstrated that DG kinase was detectable in both fractions and that substrate selectivities were the same as in the crude homogenate used in our study (Figs 2 and 3). The results obtained form the octylglycoside mixed micellar assay system suggested that the unusual species of DG, diC8, and the typical intracellular species, 18:0/20:4-DG, were competitively bound and phosphorylated by common DG kinase isozymes in intact intracellular assay systems. These characteristics may be unchanged by CCh-stimulation.

In some stimulated cell lines, PLD has been shown to contribute to PA formation through hydrolysis of major membrane phospholipids such as PtdIns and PtdCho [11]. When guinea pig taenia coli were prelabeled with 100 μM diC8 for 30 min, the diC8 was almost entirely distributed to the free DG and diC8-PA fractions (up to 85%), and was observed only slightly in PtdIns and PtdCho fractions (about 10%) (Fig. 4a). Although it is thought that endogenous [^{32}P]PA accumulation is the sum of the hydrolysis of phospholipids by PLD and the *de novo* synthesis and phosphorylation of DG by DG kinase, the accumulation of [^{32}P]diC8-PA can occur only via DG kinase under these conditions. From these results, we concluded that this diC8 can be used as a probe of agonist effects on DG kinase in guinea pig taenia coli.

In the [^{32}P]Pi- and diC8-prelabeled guinea pig taenia coli, CCh-induced stimulation was time and dose dependent (Figs 5, 6), and was blocked by the specific antagonist, Atr (Fig. 6 inset). Throughout the present study, CCh induced increases in the accumulation of [^{32}P]diC8-PA and [^{32}P]PA in a very similar manner, thus raising the possibility that DG kinase activation mediated by muscarinic receptors contributed to endogenous PA accumulation. On the other side, MacDonald *et al.* [28] investigated whether platelet-derived growth factor-induced increase in endogenous DG levels reduced the phosphorylation of exogenous short-chain DG (didecanoyl-*sn*-glycerol) in Swiss 3T3 cells. This discrepancy is not clearly understood; therefore, we concluded that effective DG kinase in guinea pig taenia coli can be variable by stimulation, and that the translocation of DG kinase to the DG-rich region or the transformation of DG kinase from its inactive to active form may be involved in increasing PA formation.

R59022, a potent DG kinase inhibitor [27], blocked the increase in accumulation of [^{32}P]diC8-PA, but [^{32}P]PA was reduced to one-half by this treatment (Fig. 7). In the mixed micellar assay system, the rates of phosphorylation of diC8 and 18:0/20:4-DG were competitively inhibited by 3 μM R59022 treatment (data not shown). These findings suggest that accumulation of [^{32}P]diC8-PA is mediated by DG kinase activation, but accumulation of [^{32}P]PA is due not only to DG kinase but also to PLD and/or *de novo* synthesis. Although these possibilities remain to be tested, DG kinase activation is coupled with receptor stimulation.

We next investigated the mechanisms of cellular DG kinase regulation. Sakane *et al.* [26] previously demonstrated that 80 KDa DG kinase is an EF-hand type calcium binding protein, and that this isozyme can be activated by calcium-dependent interaction with phospholipids in a cell-free assay system [40]. However, the role of intracellular calcium in DG kinase regulation was unknown at the tissue level. We examined the effects of intracellular calcium concentration on DG kinase activity in a tissue–DG kinase assay system. The removal of intracellular calcium accumulation by treatment with a Ca^{2+}-free Tyrode solution caused complete inhibition of CCh-induced [^{32}P]diC8-PA and [^{32}P]PA accumulation (Fig. 8). Moreover, tissues were incubated with 100 μM CCh in the presence or absence of 1 μM verapamil, and [^{32}P]diC8-PA and [^{32}P]PA accumulation was measured. In the presence of verapamil, the accumulation of both labeled species increased rapidly, returning to control levels within 5–10 min, and maximal accumulation of both types of molecules was obtained 1 min after incubation started. From these results, we concluded that the transient increase in DG kinase activation is caused by calcium released from intracellular stores, while the increased DG kinase activity was sustained by calcium influx from extracellular medium when verapamil was not present. Therefore, CCh-induced DG kinase activation may depend on both calcium sources. Thus, we next examined whether cellular DG kinase activation is triggered only by calcium concentration. However, increasing intracellular calcium by KCl treatment without receptor-mediated stimulation did not cause DG kinase activation (Fig. 9). Therefore, we concluded that the regulation of muscarinic receptor-coupled DG kinase was dependent not only on intracellular calcium but also on other intracellular factor(s).

Some reports have shown that purified DG kinase isozymes were directly activated by sphingolipids and phosphatidylserine, and Yamada *et al.* [30] found that addition of exogenous sphingosine directly activated cellular DG kinase in Jurkat cells. However, the role of these lipids in DG kinase activation in the intracellular signaling cascade is not clearly understood. On the other hand, Kanoh and Ono [43] reported that pig brain DG kinase was phosphorylated by endogenous PKC without catalysed autophosphorylation. In an attempt to establish regulators of DG kinase other than intracellular calcium, we investigated the participation of PKC on DG kinase regulation mediated by receptor stimulation. In the tissue–DG kinase assay system, DG kinase was not activated solely by 1 μM PDBu treatment as a specific PKC activator. However, in the presence of KCl, PDBu induced DG kinase activation (Fig. 9). Although the precise mechanism of PKC-associated DG kinase activation is unknown, these findings further indicate that DG kinase activation requires both increases in intracellular calcium as well as PKC activation in guinea pig taenia coli.

In the present report, we conclude that CCh

stimulates receptor-coupled DG kinase activity in guinea pig taenia coli, and that CCh-induced DG kinase activation is mediated by both increases in intracellular calcium and PKC activation. This is the first such report at the tissue level.

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