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Agonist-Mediated Activation of Phosphatidylcholine-Specific Phospholipase C and D in Intestinal Smooth Muscle

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SUMMARY

The contributions of phosphoinositide (PI)- and phosphatidylcholine (PC)-specific phospholipases [PI-specific phospholipase C (PI-PLC), PC-specific phospholipase C (PC-PLC), and phospholipase D (PLD)] to diacylglycerol (DAG) formation and regulation of the enzymes by G proteins, Ca^{2+}, and protein kinase C (PKC) were examined in dispersed intestinal circular and longitudinal muscle cells. DAG formation induced by cholecystokinin was biphasic and paralleled by PKC activity. The initial phase (~1 min) was mediated by PI-PLC in circular muscle cells and by both PI- and PC-PLC in longitudinal muscle cells, whereas the sustained phase was mediated by PC-PLC and PLD in both cell types. PC-PLC activity during the initial phase was identified by rapid formation of the initial products [^{3}H]phosphocholine (5 sec) and [^{3}H]myristate-labeled DAG (~15 sec). PLD activity did not contribute to DAG formation during the initial phase, and PI hydrolysis had no effect on PC-PLC or PLD activity during the initial or sustained phases. PLD activity during the sustained phase was evident by the formation of [^{3}H]phosphatidylethanol, a PLD-specific transphosphatidylation product. Dephosphorylation of phosphatidic acid (PA) by phosphatidate phosphohydrolase (PPH) accounted for about 50% of DAG formation; inhibition of PPH activity by propranolol or suppression of PA formation by ethanol inhibited DAG formation by 59–69% and 57–62%, respectively. Residual DAG in the presence of ethanol was augmented 55–57% by DAG kinase inhibitor, whereas residual PA was inhibited by 60–67%, implying that PA was derived from DAG, and DAG from PLC-mediated PC hydrolysis. In the presence of ethanol, calphostin C inhibited phosphatidylethanol formation but had no effect on PA or DAG levels, implying that only PLD activity was modulated by PKC. Maintenance of resting intracellular Ca^{2+} concentrations, rather than an agonist-induced increase in the intracellular Ca^{2+} concentration, was required for optimal PC-PLC and PLD activity. Guanosine-5′-*O*-(β-thio)diphosphate abolished DAG and PA formation in reversibly permeabilized muscle cells. We conclude that DAG formation in intestinal muscle is mediated by time-dependent activation of three phospholipases (PI-PLC, PC-PLC, and PLD) and two converting enzymes (DAG kinase and PPH). PC-PLC and PLD are Ca^{2+} dependent and appear to be G protein coupled; only PLD is PKC sensitive.

Ca^{2+}-mobilizing agonists that stimulate PI hydrolysis also promote the hydrolysis of other membrane phospholipids, chiefly PC (1–3). Three distinct phospholipases are involved, i.e., phospholipase A_2, which preferentially hydrolyzes phospholipids with arachidonic acid in the *sn*-2 position, to yield arachidonic acid and lyso-phospholipids (usually lyso-PC); PLC, which hydrolyzes ester bonds in the *sn*-3 position, to yield DAG and phosphocholine; and PLD, which also hydrolyzes ester bonds in the *sn*-3 position, to yield PA and choline (1–4). The primary products of PLC and PLD are interconvertible through the activity of specific kinases and phosphatases (1). DAG can be phosphorylated to PA via DAG kinase, and PA can be dephosphorylated to DAG via PPH. Similarly, choline and phosphocholine can be readily interconverted via choline kinase and phosphocholine phosphatase. These rapid interconversions imply that the activities of PC-specific enzymes cannot be inferred from the amount or composition of the products.

The relative contributions of PC-PLC and PLD to DAG formation vary greatly between cells. PLD-mediated hydrolysis of PC predominates in some cells where DAG levels are largely dependent on dephosphorylation of PA (5–10). In other cells, DAG is formed directly by PLC-mediated hydrolysis of PC (11–14). The formation of DAG is usually biphasic, with an early peak coinciding with PI hydrolysis being followed by a sustained increase reflecting PLC- and/or PLD-mediated hydrolysis of PC (4). The fatty acid composition and

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ABBREVIATIONS: PI, phosphoinositide; PI-PLC, phosphoinositide-specific phospholipase C; PC-PLC, phosphatidylcholine-specific phospholipase C; PLD, phospholipase D; PPH, phosphatidate phosphohydrolase; DAG, diacylglycerol; PA, phosphatidic acid; PEt, phosphatidylethanol; CCK-8, cholecystokinin octapeptide; GDPβS, guanosine-5′-*O*-(β-thio)diphosphate; PKC, protein kinase C; PC, phosphatidylcholine; IP_3, inositol-1,4,5-trisphosphate; PLC, phospholipase C; CCK, cholecystokinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $[Ca^{2+}]_i$, intracellular calcium concentration.

molecular species of DAG generated during the two phases reflect their derivation from distinct phospholipid substrates (5, 6, 15, 16).

The ability of PLD to catalyze a unique transphosphatidylation reaction, whereby the phosphatidyl moiety of PC is transferred to a primary alcohol to yield phosphatidylalcohol (e.g., PEt), has greatly facilitated characterization of this phospholipase and its regulation by Ca^{2+} and PKC. The absence of a comparable specific reaction has hampered characterization of PC-PLC; the involvement of PC-PLC in initial DAG formation is based on detection of the rapid formation of the primary products, phosphocholine and DAG, after preferential labeling of PC with [^{3}H]choline and [^{3}H]myristic acid, respectively.

Measurements of PLC- and PLD-mediated hydrolysis of PC in smooth muscle, using mainly cultured vascular smooth muscle cell lines, have produced evidence in favor of agonist-mediated activation of PC-PLC (prompt formation of [^{3}H]phosphocholine within seconds of stimulation by agonists) (12, 17–19) and/or PLD (prompt formation of [^{3}H]PEt and [^{3}H]myristate-labeled PA preceding that of [^{3}H]myristate-labeled DAG) (8, 10, 17, 20–22). There is substantial evidence that PLD activity is sustained and some evidence that it is dependent on extracellular Ca^{2+} and sensitive to PKC. Evidence for the participation and regulation of PC-PLC is meager (8, 10, 17, 19, 22).

There has been no systematic study of the source of DAG in visceral smooth muscle or of its formation by PLC- or PLD-mediated PC hydrolysis and its regulation by converting enzymes. The task has assumed importance with the discovery that sustained contraction induced by agonists in both vascular and visceral (gastric and intestinal) smooth muscle may be mediated by PKC. In the present study, we have used dispersed smooth muscle cells isolated separately from the circular and longitudinal muscle layers of guinea pig intestine to examine (i) the sources of DAG during the initial and sustained phases of contraction, (ii) the relative contributions of PI-PLC, PC-PLC, and converting enzymes, and (iii) the regulation of phospholipases by G proteins, PKC, and Ca^{2+}. The experimental approach involved suppression of PI-PLC activity and identification of initial PC-PLC and PLD activities by the prompt formation of the primary products of PC hydrolysis. Sustained PLD activity was identified by transphosphatidylation, and sustained PC-PLC activity was identified by direct formation of DAG after PA formation by PLD had been suppressed. The results indicate that DAG levels are determined by time-dependent and cell-specific activities of three phospholipases (PI-PLC, PC-PLC, and PLD) and two converting enzymes (PPH and DAG kinase). Both PC-PLC and PLD were G protein coupled and Ca^{2+} dependent, whereas only PLD was PKC sensitive.

Materials and Methods

Preparation of dispersed muscle cells. Muscle cells were isolated separately from the circular and longitudinal muscle layers of guinea pig intestine as described previously (23–25). Muscle strips were incubated for 30 min at 31° in a HEPES medium containing 0.1% collagenase (type II) and 0.1% soybean trypsin inhibitor. The medium consisted of 115 mM NaCl, 5.8 mM KCl, 2.1 mM KH_2PO_4, 2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 25 mM HEPES, 14 mM glucose, and 2.1% essential amino acid mixture, pH 7.4. The partly digested tissues were washed with 50 ml of enzyme-free medium, and the muscle cells were allowed to disperse spontaneously for 30 min. The cells were harvested by filtration through 500-μm Nitex filters and centrifuged twice at 350 × g for 10 min, to eliminate broken cells and organelles.

In some experiments, the dispersed cells were reversibly permeabilized as follows. The cells were incubated at 31° for 30 min in HEPES medium containing 10 mM NaCl, 140 mM KCl, 2.4 mM $MgCl_2$, 10 mM HEPES, and Trans-Port reagent (15 μl/ml), with or without GDPβS (100 μM). Permeabilization was terminated by addition of 30 μl/ml HEPES medium with 10% BSA, and the cell suspension was centrifuged for 15 min at 350 × g. The cells were resuspended in normal HEPES medium containing 0.1% bovine serum albumin and were incubated at 31° for 1 hr. The resealed cells were shown to exclude trypan blue and to contract in response to agonists and KCl (20 mM) but not to addition of 2 mM $CaCl_2$ or IP_3 (50 μM) (26).

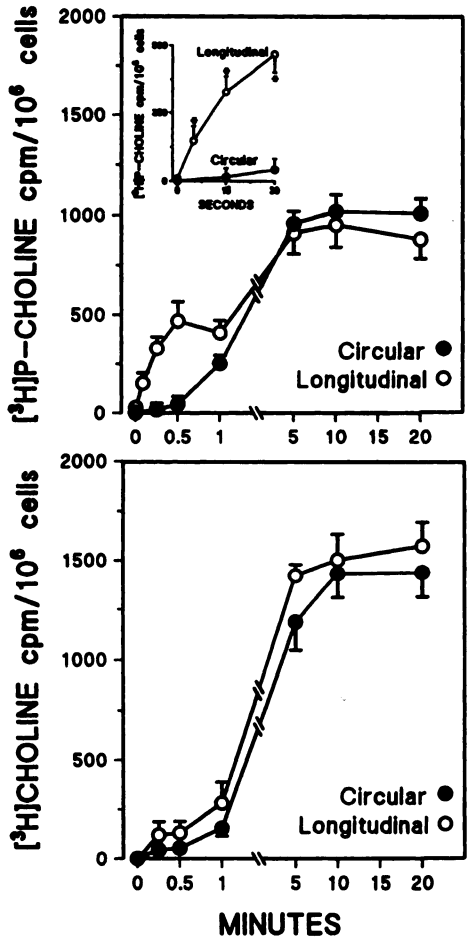

Fig. 1. Time course of CCK-induced [^{3}H]choline and [^{3}H]phosphocholine (*[^{3}H]P-choline*) formation in dispersed intestinal longitudinal and circular smooth muscle cells. Ten milliliters of cell suspension were labeled with [^{3}H]choline for 3 hr at 31°. Duplicate samples (10^6 cells/0.5 ml) were then incubated with 1 nM CCK-8 for different periods (5 sec to 20 min), in the presence or absence of 50 μM neomycin. Choline metabolites were extracted and analyzed for [^{3}H]choline and [^{3}H]phosphocholine as described in Materials and Methods. The results were expressed as cpm/10^6 cells above basal levels (basal levels in longitudinal muscle cells: [^{3}H]choline, 605 ± 129 cpm/10^6 cells; [^{3}H]phosphocholine, 774 ± 141 cpm/10^6 cells; in circular muscle cells: [^{3}H]choline, 580 ± 100 cpm/10^6 cells; [^{3}H]phosphocholine, 724 ± 106 cpm/10^6 cells). Neomycin had no effect on basal or agonist-stimulated choline or phosphocholine levels (data not shown). Phosphocholine was increased significantly within 5 sec in longitudinal muscle cells (*, $p < 0.01$). *Inset*, magnified scale. Values are means ± standard errors of four experiments.

TABLE 1

Basal and agonist-stimulated DAG, PA, and PEt formation in intestinal muscle

DAG mass and [^{3}H]myristate-labeled PA and PEt formation were measured in the basal state and during the sustained phase of stimulation of dispersed intestinal circular and longitudinal smooth muscle cells by 1 nM CCK-8. The maximal response to CCK-8 is expressed as pmol of DAG or cpm of PA or PEt per 10^6 cells above basal levels. Values are means ± standard errors of six to eight experiments.

	Basal	Maximal response
Longitudinal		
DAG mass (pmol/10^6 cells)	320 ± 30	450 ± 45
[^{3}H]PA (cpm/10^6 cells)	2319 ± 281	2552 ± 235
[^{3}H]PEt (cpm/10^6 cells)	1151 ± 160	1582 ± 231
Circular		
DAG mass (pmol/10^6 cells)	290 ± 30	444 ± 35
[^{3}H]PA (cpm/10^6 cells)	2197 ± 249	2597 ± 222
[^{3}H]PEt (cpm/10^6 cells)	1167 ± 153	1429 ± 191

Assay of [^{3}H]choline metabolites. Ten milliliters of cell suspension (2×10^6 cells/ml) were labeled with 2 μCi/ml [^{3}H]choline for 3 hr at 31°. The cells were then centrifuged at 350 × *g* for 10 min to remove excess [^{3}H]choline and were resuspended in 10 ml of fresh medium. CCK-8 (1 nM) was added to 0.5 ml of cell suspension and the mixture was incubated for various periods (5 sec to 20 min). The reaction was terminated by addition of 1.8 ml of chloroform/methanol/HCl (100:200:2, v/v/v), choline metabolites were extracted by the method of Bligh and Dyer (27), and the aqueous phase was analyzed for [^{3}H]choline and [^{3}H]phosphocholine by extraction with tetraphenylboron in heptanone (28). [^{3}H]Choline was extracted into the organic phase and [^{3}H]phosphocholine into the aqueous phase. Aliquots (250 μl) from each phase were counted for tritium content. The results were expressed as cpm/10^6 cells.

Assay of [^{3}H]PA and [^{3}H]PEt. Ten milliliters of cell suspension (2×10^6 cells/ml) were labeled with 2 μCi/ml [^{3}H]myristic acid for 3 hr at 31°. The cells were then centrifuged at 350 × *g* for 10 min, to remove excess [^{3}H]myristic acid, and were resuspended in 10 ml of fresh medium. CCK-8 (1 nM) was added to 0.5 ml of cell suspension and the mixture was incubated for various periods (15 sec to 20 min). The reaction was terminated by the addition of 1.8 ml of chloroform/methanol/HCl (100:200:2, v/v/v) and extracted by the method of Bligh and Dyer (27). The organic phase was dried under N_2 and analyzed for [^{3}H]PA by thin layer chromatography on silica gel plates (dipped in 1% potassium oxalate), with ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10) as the running solvent (29). [^{3}H]PA was identified using unlabeled standards, which were sprayed with 0.1% 1,2-dichlorofluorescein in isopropyl alcohol and visualized under UV light at 357 nm. For measurement of PEt, a specific product of PLD activity in the presence of ethanol, the cells were preincubated with 150 mM ethanol for 15 min and [^{3}H]PEt was measured as described above. The spots corresponding to PA and PEt were scraped and counted by liquid scintillation, and the results were expressed as cpm/10^6 cells.

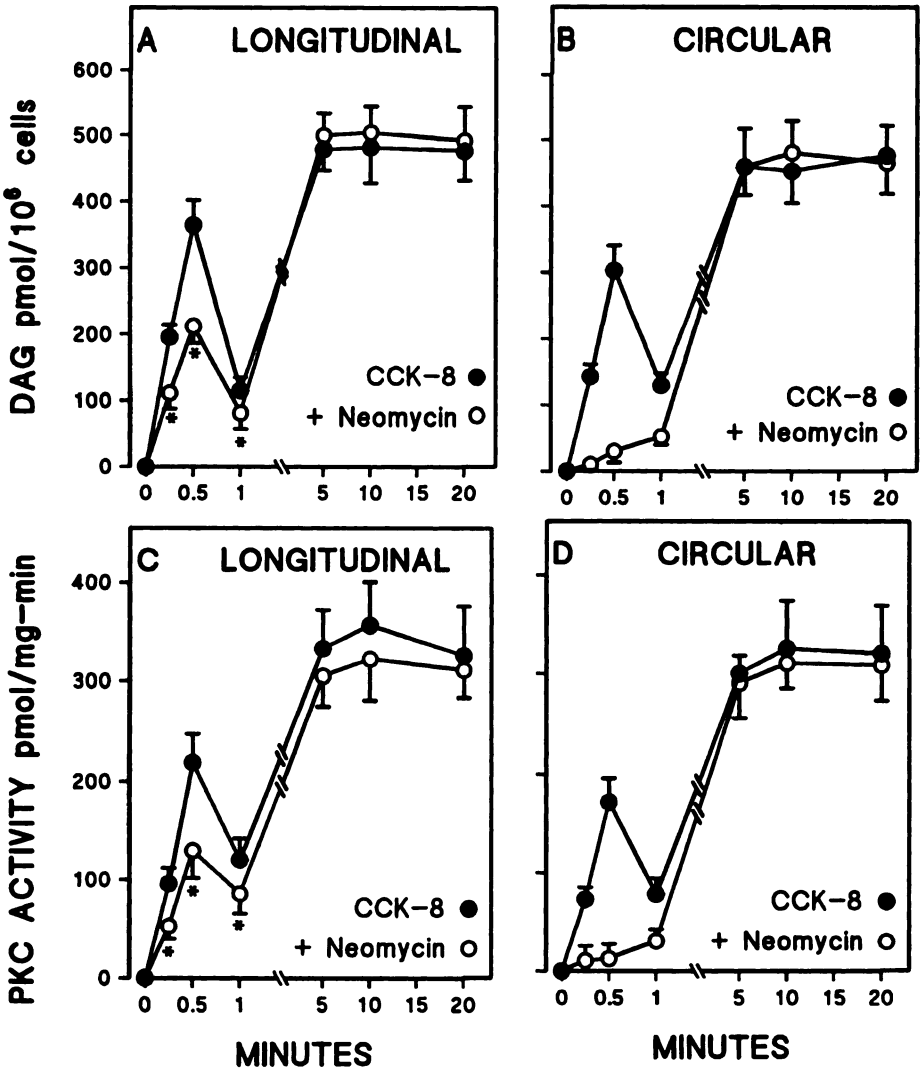

Fig. 2. Biphasic time course of CCK-induced DAG mass formation (A and B) and PKC activity (C and D) in dispersed intestinal longitudinal and circular smooth muscle cells. One milliliter of cell suspension (2×10^6 cells/ml) was incubated with 1 nM CCK for different periods (15 sec to 20 min), in the presence or absence of 50 μM neomycin, and the reaction was terminated by addition of 3 ml of chloroform/methanol (1:2, v/v) for measurement of DAG or by rapid freezing for measurement of PKC activity. DAG mass was estimated by reference to a 1,2-diolein standard curve and expressed in pmol/10^6 cells above basal levels (see Table 1 for basal levels). PKC activity was expressed as pmol/mg/min above basal levels (basal levels in longitudinal muscle cells, 150 ± 22 pmol/mg/min; in circular muscle cells, 141 ± 18 pmol/mg/min). Neomycin had no effect on basal or sustained DAG formation or PKC activity. Initial (~1-min) DAG formation and PKC activity were abolished in circular muscle cells and partly inhibited in longitudinal muscle cells. *, Significant ($p < 0.01$) residual DAG formation and PKC activity in longitudinal muscle cells after neomycin treatment. Values are means ± standard errors of six experiments for DAG and four for PKC.

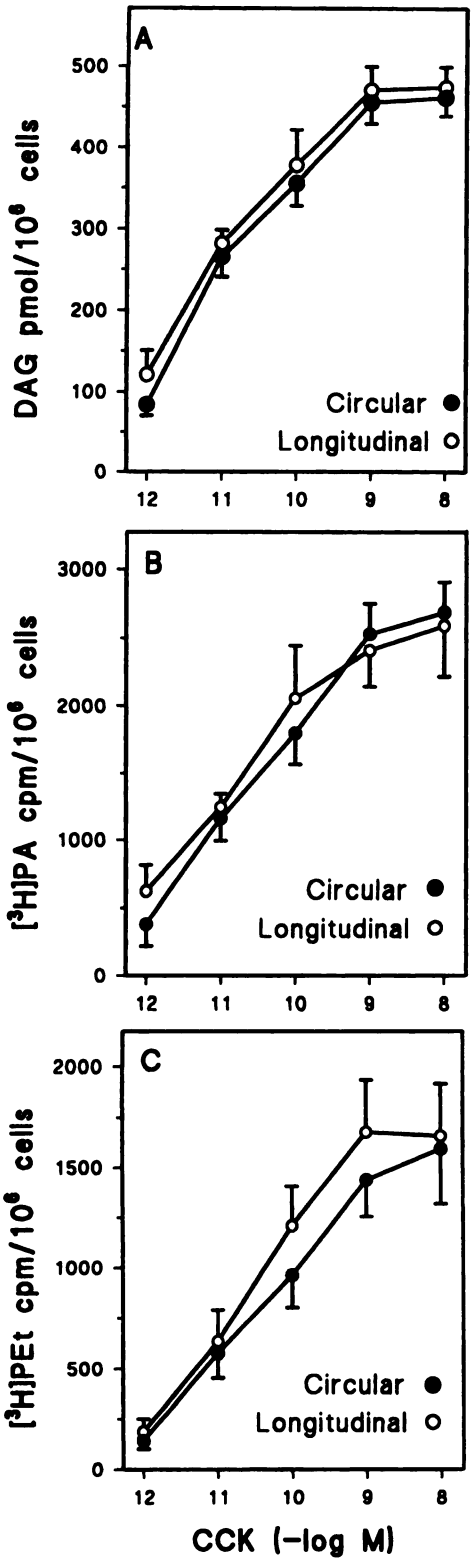

Fig. 3. Concentration-dependent stimulation of DAG, PA, and PEt formation by CCK-8 in dispersed intestinal longitudinal and circular smooth muscle cells. DAG mass and [^{3}H]myristate-labeled PA and PEt were measured as described in Materials and Methods. CCK-8 was added at different concentrations for 10 min in the presence of 50 μM neomycin. For measurement of [^{3}H]PEt, cells were preincubated with 150 mM ethanol for 15 min. DAG mass was expressed as pmol/10^6 cells above basal levels (Table 1). [^{3}H]PA and [^{3}H]PEt were expressed as cpm/10^6 cells above basal levels (Table 1). Values are means ± standard errors of four experiments.

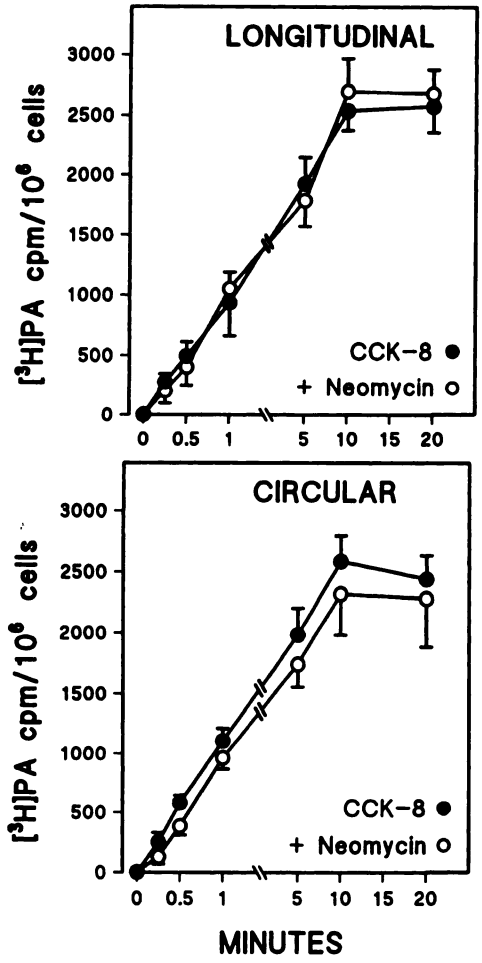

Fig. 4. Time course of CCK-induced PA formation in dispersed intestinal longitudinal and circular smooth muscle cells. Ten milliliters of cell suspension were labeled with [^{3}H]myristic acid for 3 hr at 31°. Duplicate samples (10^6 cells/0.5 ml) were then incubated with 1 nM CCK-8 for different periods (15 sec to 20 min), in the presence or absence of 50 μM neomycin. The results were expressed as cpm/10^6 cells above basal levels (Table 1). Neomycin had no effect on basal or stimulated PA levels. All values, including the increase at 15 sec, were significant ($p < 0.01$ to $p < 0.001$). Values are means ± standard errors of eight experiments.

Measurement of DAG. DAG mass was measured by the radioenzymatic method of Preiss *et al.* (30). One milliliter of cell suspension (2×10^6 cells/ml) was incubated with 1 nM CCK for different time periods and the reaction was terminated by addition of 3 ml of chloroform/methanol (1:2, v/v). After separation, 100 μl of solubilized lipid extract were incubated for 30 min with 50 μl of imidazole buffer, 10 μl of 20 mM dithiothreitol, 10 μl of 10 mM [γ-^{32}P]ATP ($2–3 \times 10^5$ cpm/nmol), and 10 μl of DAG kinase (20 milliunits). The reaction was terminated with 3 ml of chloroform/methanol (1:2, v/v); the lipids were re-extracted, dissolved in 50 μl of 5% methanol in chloroform, and spotted on silica gel plates for thin layer chromatography. DAG mass was determined by comparing [^{32}P]PA in the experimental samples with the [^{32}P]PA generated from standards containing known amounts of 1,2-diolein. The results were expressed as the increase, in picomoles/10^6 cells, compared with control.

In some experiments, myristate-labeled [^{3}H]DAG was also measured by thin layer chromatography as described above for [^{3}H]PA, except that the mobile phase consisted of petroleum ether/diethyl-ether/acetic acid (70:30:1) (12). The diglyceride bands were identified by their co-migration with authentic standards included in each lane. The results were expressed as cpm/10^6 cells above basal levels.

Measurement of PKC activity. PKC activity was measured in the particulate fraction by an adaptation of the method of Takai *et al.* (31), as described previously (24, 26). One milliliter of cell suspension (2×10^6 cells/ml) was incubated with 1 nM CCK for 60 sec, and the reaction was terminated by rapid freezing in a dry ice/acetone slurry. The cell suspension was thawed and centrifuged at $1000 \times g$ for 15 min, and the cells were resuspended in ice-cold 20 mM Tris·HCl medium, pH 7.5, containing 250 mM sucrose, 1 mM EGTA, 10 mM mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride and were then homogenized. Particulate and cytosolic fractions were separated by centrifugation and purified on DEAE-cellulose columns. PKC activity was measured by Ca^{2+}/phospholipid-dependent phosphorylation of histone-1 and was expressed as picomoles of phosphorous transferred to histone/minute/milligram of protein.

Materials. [γ-^{32}P]ATP (3000 Ci/mmol), [*methyl*-^{3}H]choline chloride (80 Ci/mmol), and [^{3}H]myristic acid (22.4 Ci/mmol) were obtained from DuPont-NEN (Boston, MA); CCK-8 from Bachem (Torrance, CA); Trans·Port reagent kit from Gibco-BRL (Grand Island, NY); calphostin C and R-59,949 [3-[2-[[bis(4-fluorophenyl)methylene]-1-piperidinyl]ethyl]-2,3-dihydro-2-thioxo-4(1*H*)-quinazolinone] from Calbiochem (San Diego, CA); DAG kinase from Lipidex (Middleton WI); cardiolipin, PEt, and PA from Avanti Polar Lipids (Alabaster, AL); collagenase type II and soybean trypsin inhibitor from Worthington (Freehold, NJ); and GDPβS, EGTA, tetraphenylboron, DL-propranolol, 3-heptanone, and all other chemicals from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis. Results were analyzed for statistical significance using Student's *t* test for paired or unpaired values. Results were expressed, per 10^6 cells, as means ± standard errors of *n* experiments; each experiment was done on cells obtained from different animals.

Results

Time course of agonist-induced [^{3}H]choline and [^{3}H]phosphocholine formation. [^{3}H]Phosphocholine formation induced by CCK-8 (1 nM) was monophasic in circular muscle cells and biphasic in longitudinal muscle cells. [^{3}H]Phosphocholine levels significantly increased (20 ± 7%, $p < 0.01$) within 5 sec of stimulation in longitudinal muscle cells, attained a peak within 30 sec, and after a partial decline increased again to a sustained 2–3-fold higher plateau (Fig. 1, *upper*). The initial peak was absent in circular muscle cells but the sustained increase was similar to that observed in longitudinal muscle cells (Fig. 1, *upper*). A gradual increase in [^{3}H]choline levels occurred in both cell types, and the magnitudes of the sustained increase were similar (Fig. 1, *lower*). The profile of [^{3}H]choline metabolites reflected the sum of [^{3}H]choline and [^{3}H]phosphocholine formation (data not shown). The results provided evidence for both early and sustained hydrolysis of PC. Neomycin, which abolishes PI hydrolysis in both cell types (32), had no effect on [^{3}H]choline or [^{3}H]phosphocholine levels, implying that PC hydrolysis was independent of PI hydrolysis. The rapid increase in [^{3}H]phosphocholine in longitudinal muscle cells suggested direct activation of a PC-PLC.

Time course and concentration dependence of agonist-induced DAG formation. Basal DAG levels were similar in the two cell types (Table 1). CCK-8 (1 nM) caused a biphasic increase of DAG in both cell types. Levels significantly increased (50 ± 3%, $p < 0.001$) within 15 sec, attained an initial peak within 30 sec, and after a partial decline increased again to a higher sustained plateau (Fig. 2, A and B). The sustained plateau was concentration dependent, with similar EC_{50} values (~8 pM) and maximal responses in the two cell types (Fig. 3A; Table 1). The initial peak was abolished by neomycin in circular muscle cells but was only partly inhibited (50 ± 6%, $p < 0.001$) in longitudinal muscle cells; neomycin had no effect on the sustained increase of DAG in either cell type (Fig. 2, A and B). The pattern of DAG formation suggested that the initial increase in longitudinal muscle was only partly dependent on PI hydrolysis and that the sustained increase in both cell types was completely independent of PI hydrolysis.

Direct evidence that the neomycin-resistant component of DAG during the initial phase of stimulation in longitudinal muscle cells was derived from PLC-mediated hydrolysis of PC was obtained from concurrent measurements of [^{3}H]myristate-labeled DAG in the presence of neomycin and propranolol (to block conversion of PA to DAG) (5, 33). Under these conditions, CCK-8 increased [^{3}H]DAG by 46 ± 7% ($p < 0.01$) at 15 sec and 83 ± 12% ($p < 0.01$) at 30 sec; no increase was observed in circular muscle cells. The results provided additional evidence for PC-PLC activity during the initial phase of stimulation in longitudinal muscle cells.

Agonist-induced activation of PKC. The time course of activation of Ca^{2+}-dependent PKC by CCK-8 closely paralleled the biphasic time course of DAG formation (Fig. 2, C and D). In cells treated with neomycin, the initial increase was abolished in circular muscle cells and was partly inhibited in longitudinal muscle cells. Neomycin had no effect on the sustained increase of PKC activity in either cell type.

Time course and concentration dependence of agonist-induced PA formation. PA formation induced by CCK-8 was monophasic in both cell types. A significant increase in [^{3}H]myristate-labeled PA occurred within 15 sec (13 ± 4% in circular muscle cells and 12 ± 3% in longitudinal

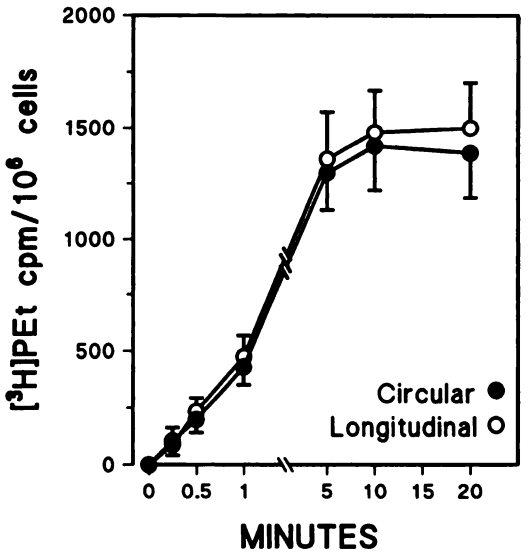

Fig. 5. Time course of CCK-induced PEt formation in dispersed intestinal circular and longitudinal smooth muscle cells. Ten milliliters of cell suspension (2×10^6 cells/ml) were labeled with [^{3}H]myristic acid for 3 hr at 31°. The cells were preincubated with 150 mM ethanol for 15 min, and duplicate samples (10^6 cells/0.5 ml) were then incubated with 1 nM CCK-8 for different periods (15 sec to 20 min). PEt was extracted and analyzed by thin layer chromatography, as described in Materials and Methods. The results were expressed as cpm/10^6 cells above basal levels (Table 1). All values, including the increase at 15 sec, were significant ($p < 0.01$ to $p < 0.001$). Values are means ± standard errors of eight experiments.

muscle cells, $p < 0.01$), and levels attained a plateau within 10 min (Fig. 4). The sustained plateau was concentration dependent, with similar EC_{50} values (~20 pM) and maximal responses in the two cell types (Fig. 3B; Table 1). Neomycin had no effect on the initial or sustained increase of PA in either cell type (Fig. 4). Because neomycin partly (longitudinal muscle) or completely (circular muscle) inhibited the initial increase of DAG, the absence of an effect on PA suggested that there was no conversion of DAG to PA during the initial period (~1 min) and that formation of PA during this period resulted from direct activation of PLD.

Time course and concentration dependence of agonist-induced activation of PLD. Treatment of muscle cells with 150 mM ethanol for 15 min before stimulation with CCK-8 elicited an increase of [^{3}H]myristate-labeled PEt in both cell types that mimicked the monophasic increase of PA (Fig. 5). Under these conditions, PLD-mediated hydrolysis of PC results in transphosphatidylation and the formation of PEt and choline. The sustained plateau was concentration dependent, with similar EC_{50} values (~38 pM) and maximal responses in the two cell types (Fig. 3C; Table 1).

Time-dependent interconversion of PA and DAG. To define the sources of DAG and PA and to determine the extent of interconversion, the two products were measured after treatment of the cells with ethanol or propranolol. Ethanol had no effect on the initial increase of DAG but inhibited the sustained increase by 61.7 ± 5.1% in circular muscle cells ($p < 0.001$) and by 57.3 ± 5.6% in longitudinal muscle cells ($p < 0.001$) (Fig. 6, A and B). The lack of effect during the initial phase implied that DAG was not derived from PA during this period. The extent of inhibition of DAG formation during the sustained phase reflected the amount of DAG derived from PA and provided evidence of sequential activation of PLD and PPH.

Measurement of PA after treatment with ethanol supported these conclusions. Ethanol virtually abolished the initial increase of PA in the two cell types, implying that it was derived exclusively from PLD-mediated hydrolysis of PC during this period. In addition, ethanol partially inhibited the sustained increase of PA, by 70.9 ± 6.0% in circular muscle cells and by 56.6 ± 4.3% in longitudinal muscle cells ($p < 0.001$) (Fig. 6, C and D). The residual PA was largely derived from DAG via DAG kinase-mediated phosphorylation. Evidence in favor of this notion was obtained by treatment of the cells with ethanol and the DAG kinase inhibitor R-59,949 (34); under these conditions, DAG levels during the sustained phase were increased by 55 ± 4% ($p < 0.01$) in circular muscle cells and by 57 ± 4% ($p < 0.01$) in longitudinal muscle

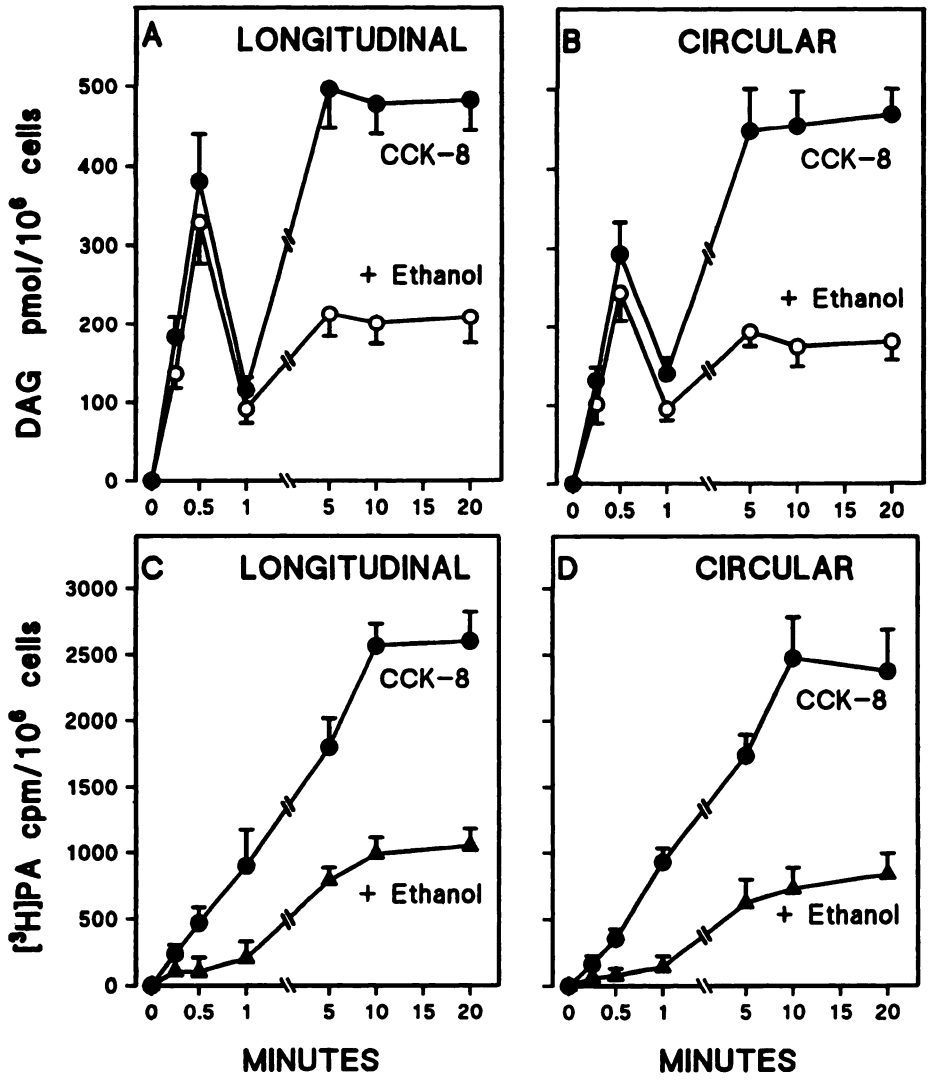

Fig. 6. Time course of CCK-induced DAG and PA formation in the presence of ethanol. One milliliter of cell suspension (2×10^6 cells/ml) was incubated with 1 nM CCK for different time periods (15 sec to 20 min), in the presence or absence of 150 mM ethanol, and the reaction was terminated by addition of 3 ml of chloroform/methanol (1:2, v/v). DAG and PA were extracted and measured as described in Materials and Methods. DAG mass was expressed as pmol/10^6 cells and PA as cpm/10^6 cells above basal levels (Table 1). Initial CCK-stimulated DAG formation was not affected by ethanol, whereas initial PA formation was abolished. Both sustained DAG and PA levels were significantly inhibited ($p < 0.01$). Values are means ± standard errors of four experiments.

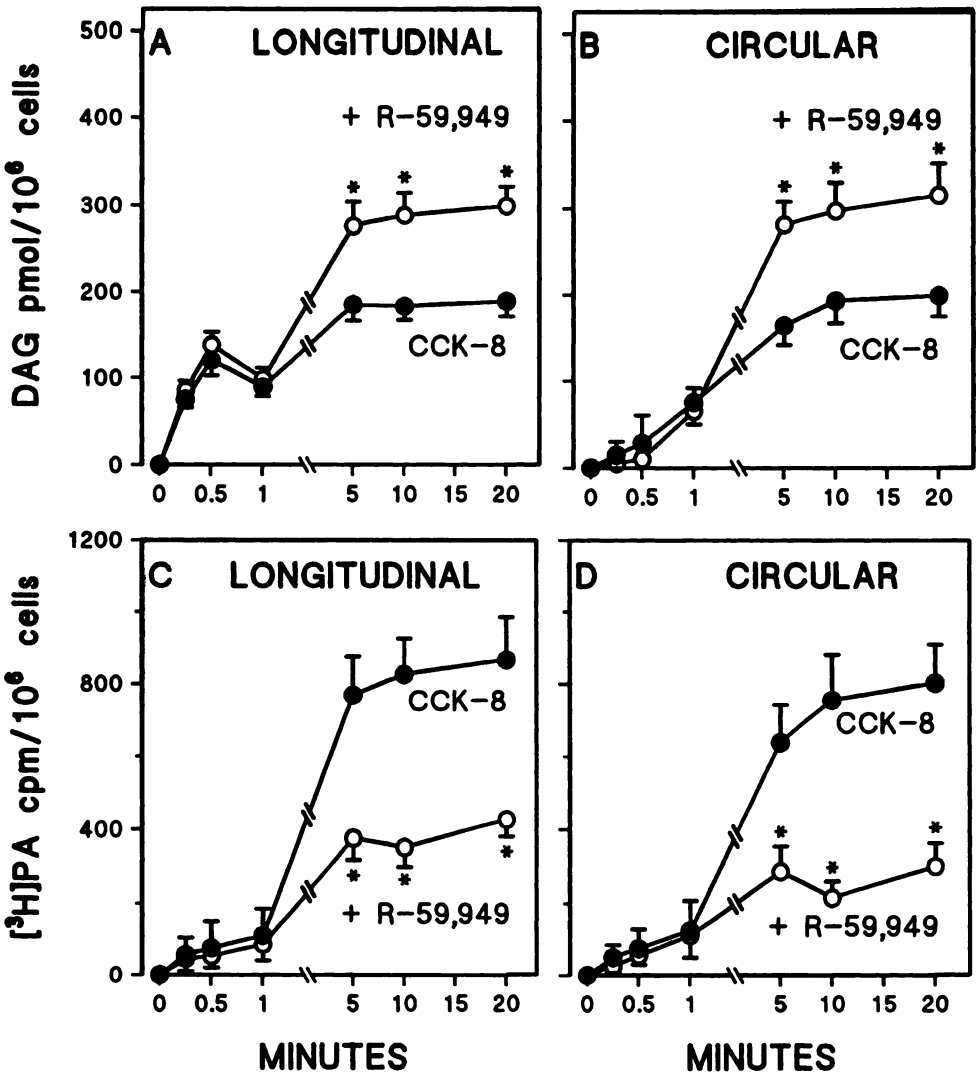

Fig. 7. Effect of the DAG kinase inhibitor R-59,949 on CCK-stimulated DAG and PA formation in the presence of ethanol. One milliliter of cell suspension (2×10^6 cells/ml) was preincubated for 15 min with 150 mM ethanol and then incubated with 1 nM CCK for different time periods (15 sec to 20 min), in the presence or absence of the DAG kinase inhibitor R-59,949 (10 μM). DAG and PA were extracted and measured as described in Materials and Methods. DAG mass was expressed as pmol/10^6 cells and PA as cpm/10^6 cells above basal levels (Table 1). *, Significant augmentation of CCK-stimulated DAG formation and inhibition of CCK-stimulated PA formation during the sustained phase ($p < 0.01$). R-59,949 had no effect on DAG or PA during the initial phase (1 min). Values are means ± standard errors of four experiments.

cells (Fig. 7, A and B), whereas PA levels were inhibited by 67 ± 2% ($p < 0.001$) in circular muscle cells and by 60 ± 1% ($p < 0.001$) in longitudinal muscle cells (Fig. 7, C and D). The increase in DAG and the decrease in PA implied that DAG was a primary product of PC-PLC activity that was partly converted to PA. The DAG kinase inhibitor had no effect on initial DAG or PA levels, suggesting that there was no interconversion of PA and DAG during the initial phase (Fig. 7).

Additional evidence for direct activation of PC-PLC and PLD as well as for interconversion of PA and DAG during the sustained phase was obtained with propranolol, a putative inhibitor of PPH (5, 33). Propranolol (300 μM) had no effect on the initial increase of PA but significantly augmented the sustained increase, by 74 ± 6% ($p < 0.01$) in circular muscle cells and by 73 ± 6% ($p < 0.01$) in longitudinal muscle cells (Fig. 8, C and D). The increase reflected the amount of PA that is normally converted to DAG by PPH. The lack of effect of propranolol on the initial increase of PA suggested that PA was not converted to DAG during this period.

Propranolol (300 μM) had no effect on the initial neomycin-resistant peak of DAG in longitudinal muscle but inhibited the sustained increase of DAG by 68.9 ± 2.5% ($p < 0.001$) in circular muscle cells and by 59.2 ± 2.8% ($p < 0.001$) in longitudinal muscle cells (Fig. 8, A and B). The inhibitory effect of propranolol was concentration dependent, with an IC_{50} of 100 μM and maximal inhibition at 300 μM in both cell types; propranolol had no effect on basal DAG formation at any concentration level (data not shown). The extent of inhibition of sustained DAG formation reflected the component of DAG derived from PPH-mediated conversion of PA to DAG and was similar in magnitude to that obtained when PA formation was suppressed with ethanol (Fig. 6).

G protein-dependent activation of PC-PLC and PLD. The pattern of DAG and PA formation in neomycin-treated muscle cells suggested that PI hydrolysis was not involved in PLC- and PLD-mediated PC hydrolysis. Direct G protein dependence of PC-PLC and PLD activity was assessed in muscle cells that had been transiently permeabilized, incubated with 100 μM GDPβS, and then resealed; in cells treated in this fashion, GDPβS selectively blocks responses to agonists and G protein activators (e.g., fluoride) (26). In the present study, both the initial and sustained increases of DAG and PA were abolished (Fig. 9). Pretreatment of the cells for 1 hr with 200 ng/ml pertussis toxin had no effect on DAG or PA levels in either cell type (data not shown).

Selective regulation of PLD activity by PKC. Treatment of the cells with the PKC inhibitor calphostin C (1 μM) (35, 36) had no effect on the initial increase of DAG or PA in

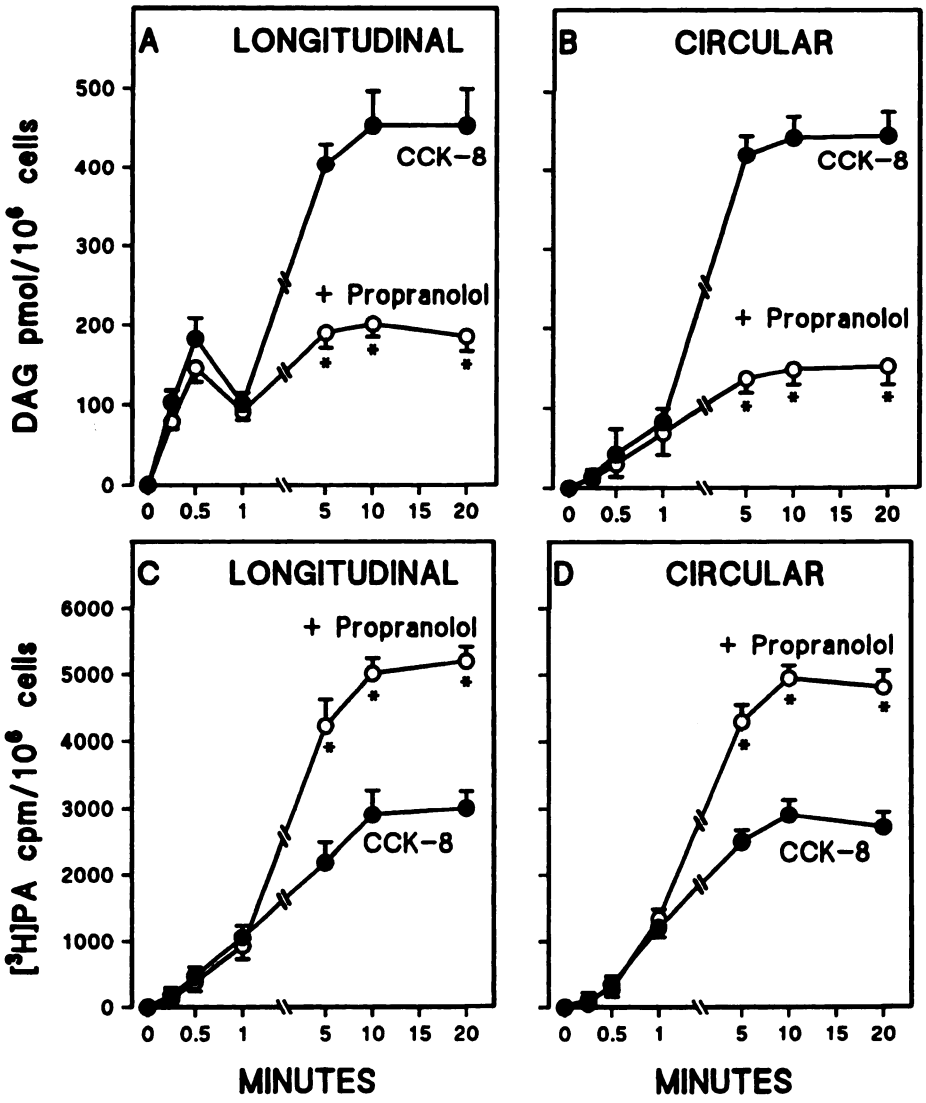

Fig. 8. Time course of DAG and PA formation in the presence of propranolol. One milliliter of cell suspension (2×10^6 cells/ml) was incubated for 15 min with 300 μM propranolol and then incubated with 1 nM CCK for different time periods (15 sec to 20 min). DAG and PA were extracted and measured as described in Materials and Methods. DAG mass was expressed as pmol/10^6 cells and PA as cpm/10^6 cells above basal levels (Table 1). *, Significant augmentation of CCK-stimulated PA formation and inhibition of CCK-stimulated DAG formation during the sustained phase ($p < 0.01$). Propranolol had no effect on DAG or PA formation during the initial phase (1 min). Values are means ± standard errors of four experiments.

neomycin-treated cells but inhibited the sustained increase by 21 ± 6% ($p < 0.05$) in circular muscle cells and by 25 ± 8% ($p < 0.05$) in longitudinal muscle cells (Fig. 10). It could not be ascertained from these results whether PKC exerted its effect directly on PC-PLC, PLD, and/or the converting enzymes. To gain additional insight into the locus of action of PKC, the experiments were repeated in cells that had been pretreated with ethanol and neomycin. In these cells, calphostin C inhibited the sustained increase of PEt by 20 ± 6% ($p < 0.05$) in circular muscle cells and by 23 ± 5% ($p < 0.02$) in longitudinal muscle cells but had no effect on DAG or PA levels (Table 2). The effect on PEt levels implied that PLD activity was sensitive to PKC, whereas the activities of PC-PLC, PPH, and DAG kinase, which were jointly responsible for residual PA and DAG levels, were insensitive to PKC.

Role of Ca^{2+} in PLC- and PLD-mediated hydrolysis of PC. Previous studies showed that neomycin abolishes agonist-induced Ca^{2+} mobilization in circular muscle cells, which is mediated by IP_3-dependent Ca^{2+} release (23, 32). In contrast, neomycin has no effect on Ca^{2+} mobilization in longitudinal muscle cells, where PI metabolism reflects preferential hydrolysis of phosphatidylinositol-4-monophosphate and generation of inositol bisphosphate and DAG (26). Ca^{2+} mobilization in longitudinal muscle cells is mediated by agonist-induced Ca^{2+} influx, which triggers Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores (23, 37).

Treatment of circular muscle cells with neomycin abolished the initial component of DAG formation, which is wholly derived from PI hydrolysis, but had no effect on initial or sustained PA or PEt formation (Figs. 2 and 4). Thus, agonist-induced IP_3-dependent Ca^{2+} mobilization was not a prerequisite for PC hydrolysis. Similarly, treatment of longitudinal muscle cells with the Ca^{2+} channel blocker methoxyverapamil in the presence of neomycin had no effect on initial or sustained DAG or PA formation (data not shown). Thus, agonist-induced Ca^{2+} mobilization in longitudinal muscle also was not a prerequisite for PC hydrolysis.

However, when the cells were incubated for 30 min in Ca^{2+}-free medium (0 mM Ca^{2+} and 2 mM EGTA) in the presence of neomycin, the sustained increase of PA and DAG was inhibited by 40–50% in circular and longitudinal muscle cells. In the presence of ethanol, the increase of [^{3}H]PEt, which reflects PLD activity, and the residual increases of PA and DAG, which reflect mainly PC-PLC activity, were inhibited by about 30% in both cell types (Table 2). The experimental conditions (plus neomycin, in Ca^{2+}-free medium)

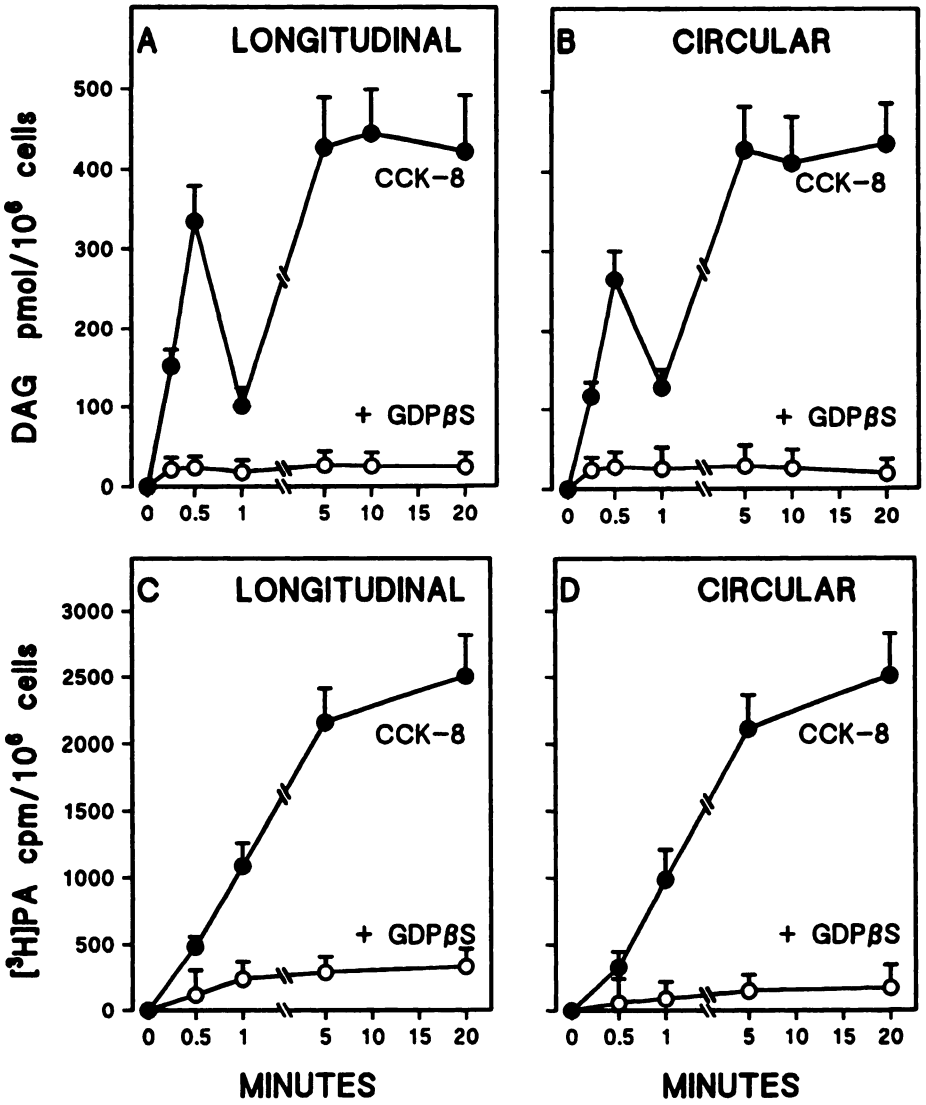

Fig. 9. G protein dependence of CCK-induced DAG and PA formation in dispersed longitudinal and circular smooth muscle cells. Dispersed cells were reversibly permeabilized with Trans-Port reagent in Ca^{2+}- and Mg^{2+}-free HEPES medium, in the presence or absence of GDPβS (100 μM). For measurement of PA, the cells were labeled with [^{3}H]myristic acid, as described in Materials and Methods, and incubated with 1 nM CCK-8 for different periods (15 sec to 20 min). DAG mass was expressed as pmol/10^6 cells and PA as cpm/10^6 cells above basal levels (Table 1). Values are means ± standard errors of four experiments.

were designed not only to prevent Ca^{2+} mobilization in both cell types but also to cause a substantial decrease (~50%) in resting $[Ca^{2+}]_i$ (control resting $[Ca^{2+}]_i$ in circular and longitudinal muscle cells, 62 ± 4 nM and 58 ± 8 nM, respectively; resting $[Ca^{2+}]_i$ in Ca^{2+}-free medium, 30 ± 3 nM and 27 ± 2 nM, respectively). The results suggested that maintenance of resting $[Ca^{2+}]_i$, rather than an increase in $[Ca^{2+}]_i$, was necessary for optimal PC-PLC and PLD activity.

Discussion

This study shows that agonist-induced stimulation of DAG in intestinal circular and longitudinal smooth muscle cells is mediated by time-dependent activation of three phospholipases, PI-PLC, PC-PLC, and PLD, and two converting enzymes, PPH and DAG kinase. Although labeled as discrete entities, PC-PLC or PLD could encompass more than one enzyme. A model depicting the pathways involved is shown in Fig. 11.

The increase in DAG was biphasic in both cell types; the initial phase was brief (~1 min) and was mediated by PI-PLC alone in circular muscle cells and by both PI-PLC and PC-PLC in longitudinal muscle cells. Neither PPH nor DAG kinase was involved in modulating the levels of DAG during this phase. The participation of PC-PLC in longitudinal muscle cells was evident in the rapid formation of [^{3}H]phosphocholine and [^{3}H]myristate-labeled DAG, the primary co-products of PLC-mediated hydrolysis of PC, within 5–15 sec of stimulation by CCK-8. Although PLD was rapidly activated in both cell types, as shown by the rapid formation of [^{3}H]myristate-labeled PA (or [^{3}H]PEt in the presence of ethanol), the increase in PA did not contribute to initial DAG levels. Suppression of PA formation by ethanol had no effect on DAG levels in either cell type. Blockade of PPH activity by propranolol and of DAG kinase activity by R-59,949 had no effect on DAG or PA levels in either cell type, confirming the absence of PPH or DAG kinase activity during the initial phase. The absence of early conversion of PA to DAG was previously noted in some studies of cultured vascular smooth muscle cells, where formation of [^{3}H]myristate-labeled PA was shown to precede that of [^{3}H]myristate-labeled DAG (8, 10, 17, 20–22). Other studies of cultured vascular muscle cells, however, showed rapid activation of both PC-PLC and PLD, with concurrent formation of PA and DAG (8, 10, 12, 17–22).

Neomycin was previously shown to inhibit PI hydrolysis, IP_3 formation, and IP_3-dependent Ca^{2+} release in intestinal

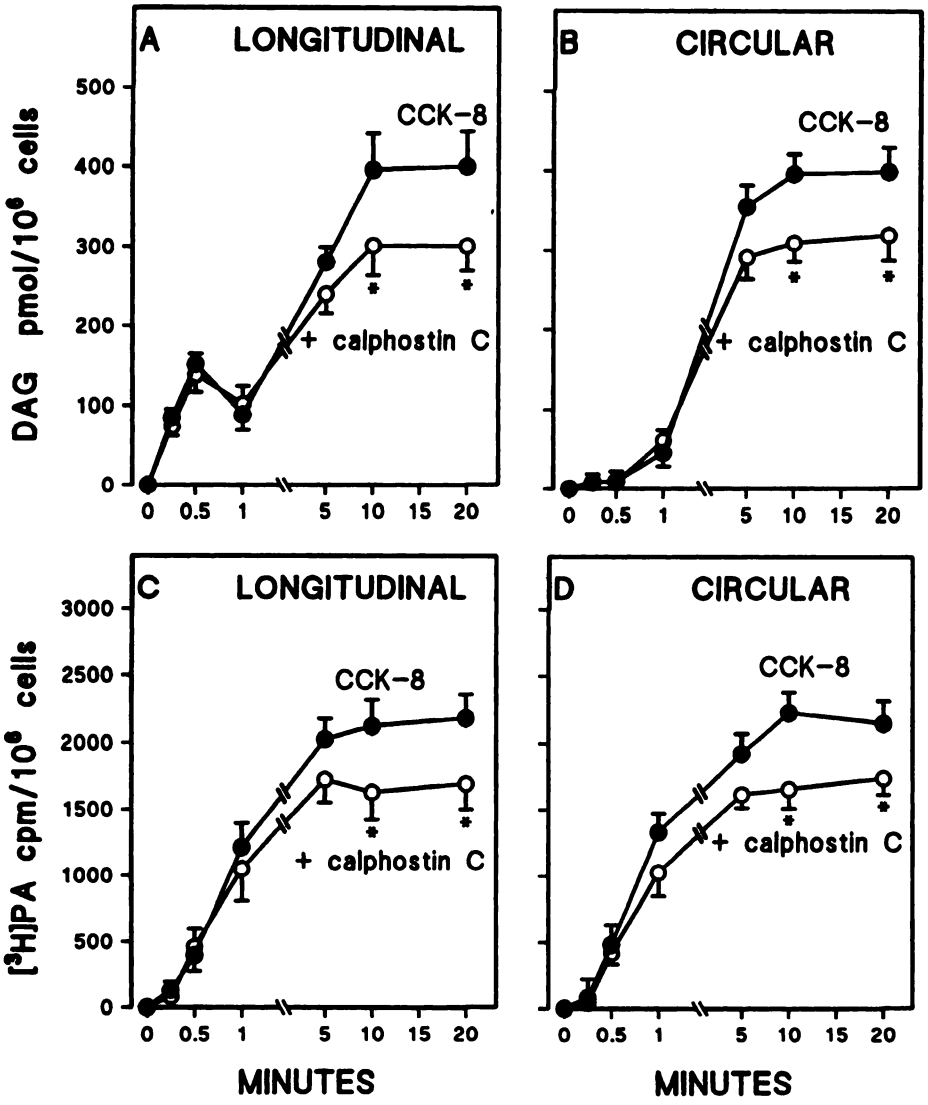

Fig. 10. Effect of calphostin C on CCK-induced DAG and PA formation in dispersed intestinal circular and longitudinal smooth muscle cells. One milliliter of cell suspension (2×10^6 cells/ml) was incubated with 1 nM CCK for different time periods (15 sec to 20 min), in the presence or absence of 1 μM calphostin C. DAG and PA were extracted and measured as described in Materials and Methods. DAG mass was expressed as pmol/10^6 cells and PA as cpm/10^6 cells above basal levels (Table 1). *, Significant inhibition of sustained DAG and PA formation. Calphostin C had no effect on DAG or PA levels during the initial phase (1 min). Values are means ± standard errors of four experiments.

TABLE 2

Inhibition of DAG, PA, and PEt formation in intestinal muscle by calphostin C and in Ca^{2+} free-medium

Experiments were done separately with dispersed intestinal circular and longitudinal smooth muscle cells after 15-min treatment with 150 mM ethanol, in the presence of 50 μM neomycin. The results represent percentage inhibition of CCK-induced sustained formation of DAG mass and [^{3}H]myristate-labeled PA and PEt by the PKC inhibitor calphostin C and in Ca^{2+} free-medium. Values are means ± standard errors of four experiments.

	Inhibition			
	0 Ca^{2+}/2 mM EGTA		1 μM Calphostin C	
	Longitudinal	Circular	Longitudinal	Circular
	%			
DAG mass	30 ± 6 ($p < 0.01$)	28 ± 8 ($p < 0.05$)	9 ± 12 (NS)[a]	9 ± 7 (NS)
[^{3}H]PA	32 ± 7 ($p < 0.02$)	26 ± 8 ($p < 0.05$)	4 ± 7 (NS)	3 ± 12 (NS)
[^{3}H]PEt	32 ± 7 ($p < 0.02$)	37 ± 9 ($p < 0.02$)	23 ± 5 ($p < 0.02$)	20 ± 6 ($p < 0.05$)

[a] NS, not significant.

muscle cells (32). In the present study, during the initial phase this compound inhibited DAG formation and PKC activity, which are completely dependent on PI hydrolysis in circular muscle cells and partly dependent in longitudinal muscle cells. However, neomycin had no effect on products of PC hydrolysis (PA, PEt, choline, and phosphocholine) during the initial or sustained phase in either cell type, implying that neither PC-PLC nor PLD activity was dependent on PI hydrolysis.

During the sustained phase, the levels of [^{3}H]phosphocholine and DAG and those of [^{3}H]choline and PA were determined by their formation as primary products of PC-PLC and PLD activity, respectively, as well as by their interconversion. Unlike the initial phase, where [^{3}H]phosphocholine was formed as a primary product in longitudinal muscle cells only, the levels of choline metabolites during the sustained phase changed in parallel with each other. Because choline and phosphocholine are readily interconverted, their abun-

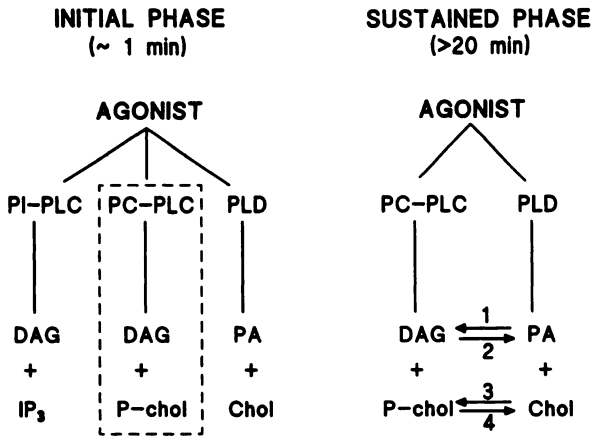

Fig. 11. Model depicting agonist-mediated, time-dependent activation of PI-PIC, PC-PLC, and PLD in intestinal circular and longitudinal smooth muscle cells. Activation of PI-PLC was transient, whereas activation of PC-PLC and PLD was sustained. The only difference between the two cell types was the absence of early activation of PC-PLC in circular muscle (*dotted lines*). Interconversion of PC products occurred only during the sustained phase. Converting enzymes were PPH (*1*), DAG kinase (*2*), choline kinase (*3*), and phosphocholine phosphatase (*4*). *P-chol*, phosphocholine; *Chol*, choline.

dance provided evidence for continued PC hydrolysis but not evidence for their formation as primary products of PLD (choline) or PC-PLC (phosphocholine). Continued PLD activity, however, was evident in the sustained formation of PEt in both cell types. Continued PC-PLC activity was demonstrable after direct formation of PA by PLD was suppressed with ethanol; under these conditions, the residual PA was derived from DAG, a primary product of PC-PLC activity, inasmuch as PA levels were strongly inhibited (60–67%), whereas DAG levels were augmented (55–57%), by the DAG kinase inhibitor R-59,949. The decrease in DAG levels induced by ethanol provided an estimate of the amount of DAG normally derived from PA, whereas DAG levels in the presence of both ethanol and R-59,949 provided an estimate of the amount of DAG derived directly from PLC-mediated hydrolysis of PC. These estimates suggest that the amount of DAG generated by activation of PC-PLC was about equal to that generated indirectly by sequential activation of PLD and PPH. These results provide direct evidence for participation of PC-PLC in sustained DAG formation in smooth muscle. Earlier studies on vascular smooth muscle showed only transient activation of PC-PLC during the initial phase and not the sustained phase of DAG formation (8, 10, 12, 17–22).

The effect of propranolol provided further support for the existence of both PLD and PC-PLC activities. Propranolol decreased DAG levels, implying that DAG was partly derived from PPH-mediated PA hydrolysis, and increased PA levels, implying that PA was partly converted to DAG. The results obtained with propranolol differ from those reported by Lassegue *et al.* (8) in cultured vascular smooth muscle cells, where propranolol inhibited agonist-stimulated DAG formation but augmented basal DAG levels. In the present study, propranolol inhibited CCK-stimulated DAG formation in a concentration-dependent fashion but had no effect on basal DAG levels; this made it possible to use propranolol as a tool with which to examine indirect generation of DAG from PA.

PKC has been shown to regulate PLD activity in various cell types (38–41); however, studies on its role in vascular smooth muscle have yielded conflicting results (4). In some but not all studies, inhibition of PKC activity or down-regulation of PKC by prolonged incubation with phorbol esters inhibited PLD activity (i.e., decreased PEt formation) (8, 21). In the present study, calphostin C in the presence of ethanol caused a decrease in PEt but not in PA or DAG. The pattern implied that only PLD activity was sensitive to PKC. Furthermore, because under these conditions (i.e., in the presence of ethanol) the residual DAG responsible for activation of PKC is derived from PC-PLC activity, the inhibitory effect of calphostin C on PEt suggests that PLD activity can be regulated indirectly by PC-PLC.

Studies on the Ca^{2+} requirement for PC-PLC and PLD activity in smooth muscle cells have also yielded conflicting results (4, 8, 10, 17, 19, 21, 22). In the present study, neither PC-PLC nor PLD activity appeared to depend on agonist-induced increases in $[Ca^{2+}]_i$. Neomycin, which abolished IP_3-dependent Ca^{2+} mobilization in circular muscle cells (32), and methoxyverapamil, which abolished Ca^{2+} mobilization in longitudinal muscle cells by blocking Ca^{2+} influx and thus Ca^{2+}-induced Ca^{2+} release (23, 37), had no effect on the sustained formation of DAG, PA, and PEt. However, a decrease in resting $[Ca^{2+}]_i$ brought about by incubation of the muscle cells in Ca^{2+}-free medium partly inhibited sustained PEt, DAG, and PA formation, implying that maintenance of resting $[Ca^{2+}]_i$, rather than an agonist-mediated increase, was necessary for optimal activity of PLD and PC-PLC. A similar effect was reported by Lassegue *et al.* (8, 19) in studies of cultured vascular smooth muscle. In the present study as well as in other studies (8, 10, 17), incubation in Ca^{2+}-free medium had no detectable effect on any PC product formed during the initial phase.

Insertion of GDPβS into reversibly permeabilized muscle cells provided an opportunity to examine the involvement of G proteins in PLC- and PLD-mediated hydrolysis of PC. GDPβS abolished initial and sustained DAG and PA formation. By comparison, neomycin inhibited only the component of the initial phase of DAG formation mediated by PI-PLC. The effect of GDPβS implied that agonist-mediated activation of PI-PLC, PC-PLC, and PLD was G protein dependent. The G protein involved in CCK-mediated PC hydrolysis was pertussis toxin insensitive. Whether the same G protein or distinct G proteins are coupled to all three phospholipases remains to be determined.

The parallelism between DAG levels and PKC activity during the initial and sustained phases implies that DAG, independently of its source or molecular composition, was effective in activating PKC. However, because only Ca^{2+}-dependent PKC activity was measured, a more precise evaluation would require characterization of Ca^{2+}-dependent and -independent PKC isoforms, to determine whether the species of DAG derived from PC hydrolysis preferentially activate specific PKC isoforms. This is particularly important during the sustained phase, where a role for Ca^{2+}-independent PKC in regulating sustained contraction of smooth muscle has been proposed (42, 43). Preliminary studies (44) confirm that a Ca^{2+}-independent PKC isoform (PKC-ε) is translocated during sustained contraction of intestinal circular smooth muscle cells; when these cells are permeabilized and incubated in a cytosol-like medium (100 nM Ca^{2+}), sustained contraction induced by CCK-8 is selectively blocked by antibodies to PKC-ε but not by antibodies to Ca^{2+}-dependent isoforms.

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