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Sustained Diacylglycerol Accumulation Resulting From Prolonged G Protein-Coupled Receptor Agonist-Induced Phosphoinositide Breakdown in Hepatocytes

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Abstract Studies in various cells have led to the idea that agonist-stimulated diacylglycerol (DAG) generation results from an early, transient phospholipase C (PLC)-catalyzed phosphoinositide breakdown, while a more sustained elevation of DAG originates from phosphatidylcholine (PC). We have examined this issue further, using cultured rat hepatocytes, and report here that various G protein-coupled receptor (GPCR) agonists, including vasopressin (VP), angiotensin II (Ang.II), prostaglandin $F_{2\alpha}$, and norepinephrine (NE), may give rise to a prolonged phosphoinositide hydrolysis. Preincubation of hepatocytes with 1-butanol to prevent conversion of phosphatidic acid (PA) did not affect the agonist-induced DAG accumulation, suggesting that phospholipase D-mediated breakdown of PC was not involved. In contrast, the GPCR agonists induced phosphoinositide turnover, assessed by accumulation of inositol phosphates, that was sustained for up to 18 h, even under conditions where PLC was partially desensitized. Pretreatment of hepatocytes with wortmannin, to inhibit synthesis of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate (PIP_2), prevented agonist-induced inositol phosphate and DAG accumulation. Upon VP stimulation the level of PIP_2 declined, but only transiently, while increases in inositol 1,4,5-trisphosphate ($InsP_3$) and DAG mass were sustained, suggesting that efficient resynthesis of PIP_2 allowed sustained PLC activity. This was confirmed when cells were pretreated with wortmannin to prevent resynthesis of PIP_2. Furthermore, metabolism of $InsP_3$ was rapid, compared to that of DAG, with a more than 20-fold difference in half-life. Thus, rapid metabolism of $InsP_3$ and efficient resynthesis of PIP_2 may account for the larger amount of DAG generated and the more sustained time course, compared to $InsP_3$. The results suggest that DAG accumulation that is sustained for many hours in response to VP, Ang.II, NE, and prostaglandin $F_{2\alpha}$ in hepatocytes is mainly due to phosphoinositide breakdown. J. Cell. Biochem. 94: 389–402, 2005.

Key words: diacylglycerol; phospholipase C; phospholipase D; hepatocytes; G protein-coupled receptors

A large number of agents regulate cell function by activating various isoforms of phosphoinositide-specific phospholipase C (PLC), which hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2), with the resultant rapid formation of diacylglycerol (DAG), along with inositol 1,4,5-trisphosphate ($InsP_3$) [Berridge, 1993]. However, while this mechanism seems to explain the acute, transient increase in DAG, a more prolonged elevation, which may be of importance in regulation of various aspects of cell function including proliferation, is generally believed to result largely from phosphatidylcholine (PC) breakdown [Exton, 1990; Nishizuka, 1995; Quest et al., 1996].

The main arguments against a role of phosphoinositide hydrolysis by PLC as a source of sustained DAG elevation is that rapid desensitization seems to be a feature of all receptors linked to PLC [Wojcikiewicz et al., 1993; Fisher, 1995], and that PIP_2, the substrate of PLC, is rapidly depleted [Augert et al., 1989a; Exton, 1997]. However, there are data suggesting that receptor desensitization may be only partial, and that phosphoinositide hydrolysis may be sustained [Wojcikiewicz and Nahorski, 1991; Tobin et al., 1992; Allan and Exton, 1993; Willars et al., 1998a,b], and it is, therefore, still not clarified to what extent PLC-catalyzed

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generation of DAG from PIP_2 can continue as a long term response to receptor stimulation.

The best characterized route of DAG generation from PC involves phospholipase D (PLD), generating phosphatidic acid (PA), which can then be converted to DAG by phosphatidate phosphohydrolase [Hammond et al., 1995; Exton, 1997], however, the signaling role of PLD-derived DAG has been questioned [Pettitt et al., 1997; Wakelam, 1998]. Other data suggest that DAG may also be formed directly from PC by PC-specific PLC (PC-PLC), [Cheng et al., 1997; van Dijk et al., 1997], although the existence of such enzyme(s) in mammalian cells is disputed, and it has been proposed that DAG formation ascribed to PC-PLC might be accounted for by sphingomyelin synthase activity [Luberto and Hannun, 1998]. The source of DAG in sustained responses is therefore not clarified.

In previous studies examining the role of PLC in the growth-promoting effects of GPCR agonists, such as vasopressin (VP), angiotensin II (Ang.II), norepinephrine (NE) and prostaglandin $F_{2\alpha}$, we have observed that DAG elevation can occur in the absence of significant increases in $InsP_3$ mass, and that agonist-induced DAG accumulation was more prolonged than $InsP_3$ accumulation [Dajani et al., 1999; Sandnes et al., 1999]. The aim of the present study was to examine the pathways of DAG formation, and in particular whether GPCR agonist-induced activation of PLC can account for the sustained accumulation of DAG in hepatocytes. We have found no evidence of PC-derived DAG formation, but show that phosphoinositide turnover is sustained for up to18 h after agonist addition, and that rapid metabolism of $InsP_3$, compared to that of DAG, may account for the different amounts of $InsP_3$ and DAG generated. These results suggest that phosphoinositide hydrolysis is the main source of sustained DAG accumulation in response to GPCR activation in hepatocytes.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), William's medium E, penicillin, and streptomycin were from Gibco (Grand Island, NY). Prostaglandin $F_{2\alpha}$ (tris salt), NE bitartrate, VP, Ang.II, [des-Gly^9-β-mercapto-β,β-cyclopentamethylenepropionyl^1 O-Et-Tyr^2,Val^4, Arg^8]-VP, timolol maleate, dexamethasone, collagenase, fatty acid-free albumin, collagen, *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid (HEPES), 12-O-tetradecanoylphorbol 13-acetate (TPA), and wortmannin were from Sigma Chemical Co. (St. Louis, MO). A23187 was from Calbiochem (San Diego, CA). D-myo[^{3}H]inositol-1,4,5-trisphosphate (21.0 Ci/mmol) and [9,10-^{3}H(N)]palmitic acid were from DuPont NEN (Boston, MA) [γ^{32}P]-ATP (3,000 Ci/mmol) and myo-[2-^{3}H]inositol (15.0 Ci/mmol) were from Amersham Pharmacia Biotech (Amersham, Buckinghamshire, UK). All other chemicals were of analytical grade.

Isolation and Culture of Hepatocytes

Male Wistar rats (170–220 g), fed ad libitum, were used. Hepatocytes were isolated by in vitro collagenase perfusion and low speed centrifugations [Berry and Friend, 1969; Seglen, 1976], with modifications as previously described [Christoffersen et al., 1984]. Cell viability, measured as the ability to exclude trypan blue, was 90–97%. The cells were suspended in culture medium and plated at a density of 20,000/cm^2 in Costar wells (10 cm^2). The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium and William's medium E; it was bicarbonate buffered and supplemented with penicillin (67 μg/ml), streptomycin (100 μg/ml), insulin (100 nM), dexamethasone (25 nM), and collagen (3 μg/ml).

Assay of DAG

Methanol (0.5 ml) was added to the cultures, the cells were collected with a rubber policeman, and the wells were washed with 0.5 ml methanol. The combined methanol phase was mixed with 0.5 ml chloroform and 0.4 ml 1 M NaCl and vigorously vortexed. Phases were separated by addition of 0.5 ml chloroform and 0.5 ml 1 M NaCl, followed by vortexing and centrifugation at 2,000*g* for 5 min. An aliquot of the chloroform layer was evaporated in a SpeedVac centrifuge and analyzed within 48 h. The DAG mass in the chloroform layer was analyzed by enzymatic conversion to PA in the presence of [γ^{32}P]-ATP, essentially as described [Preiss et al., 1987].

Assay of PLD

Hepatocytes were cultured for 3 h, with 5 μCi/ml [^{3}H]palmitic acid added at the time of plating. Thirty minutes before agonist addition, cells were washed twice with Krebs–Ringer–

HEPES buffer (containing in mM: NaCl 119.0; KCl 3.0, KH_2PO_4 2.4, $MgSO_4$ 1.2, $CaCl_2$ 2.0, HEPES 25.0) with 0.5% albumin and 10 mM glucose, and cells were subsequently incubated in the same buffer without albumin. 1-butanol (30 mM final concentration) was added to the cells 5 min prior to agonist stimulation, which was carried out for 15 min. The reaction was stopped by removing the buffer and adding 0.5 ml methanol. Lipids were extracted as described above for DAG, and an aliquot of the chloroform phase was evaporated in a SpeedVac centrifuge. The dried lipid was dissolved in 50 μl chloroform containing 10 μg each of PA and phosphatidylbutanol, and applied on TLC plates. The plates were developed in the upper phase of ethylacetate/isooctane/acetic acid/water 65:10:15:50 (v/v). The spots corresponding to phosphatidylbutanol were visualized under UV light after spraying the plates with primuline. Radioactivity was determined by scintillation counting after excision.

Assay of Inositol 1,4,5-Trisphosphate

After culturing for 3 h, medium was removed, and Krebs–Ringer–HEPES buffer pH 7.4, containing 10 mM glucose, was added. Cells were preincubated in buffer for 30 min before agonist stimulation. The reaction was stopped by removing the buffer and adding 0.4 M ice-cold perchloric acid. Cells were scraped with a rubber policeman, and the samples were centrifuged at 1,250*g* for 10 min at 4°C. The pellet was used for extraction of PIP_2, as described below. The supernatant was neutralized with 4 M KOH, 1 M Tris, 60 mM Hepes, in the presence of Universal indicator, and samples were centrifuged at 1,250*g* for 10 min at 4°C. The $InsP_3$ content of the supernatant was determined by a competitive radioligand binding assay [Palmer et al., 1989].

Extraction of Phosphatidylinositol 4,5-Bisphosphate and Conversion to Inositol 1,4,5-Trisphosphate

The pellet obtained after centrifugation of PCA-precipitated cells, as described above for inositol 1,4,5-trisphosphate, was washed once with 1 ml 0.4 M perchloric acid containing 1 mM EDTA, and once with 1 ml distilled water. Lipids were extracted according to Schacht [1981], using 1.5 ml chloroform/methanol 1:2 (v/v) and vigorous vortexing. Then 0.5 ml chloroform and 0.5 ml 2.4 N HCl was added, samples were vortexed and centrifuged at 1,250*g* for 10 min. The chloroform phase was transferred to fresh tubes, and the upper layer was reextracted with 1 ml chloroform. The pooled chloroform phases were washed once with 2 ml methanol/1 N HCl (1:1 v/v). An aliquot of the chloroform phase was evaporated in a SpeedVac centrifuge. To the dried lipids 300 μl methylamine solution (43 ml 40% methylamine in water, 46 ml methanol, 11 ml 1-butanol) was added, and samples were deacylated at 53°C for 1 h [Clarke and Dawson, 1981]. The samples were evaporated in a Speed-Vac centrifuge. The dried samples were dissolved in water, and glycerol moieties were removed by incubating with 50 mM sodium periodate for 30 min at room temperature. The reaction was stopped using 10% ethylene glycol for 15 min, followed by 0.3% (v/v) dimetylhydrazine (pH 7) for 3 h [Irvine et al., 1985]. The resulting $InsP_3$ was assayed by competitive radioligand binding [Palmer et al., 1989].

Inositol Phosphate Accumulation

Hepatocytes were cultured for 3 h as described above, with 5 μCi/ml myo-[2-^{3}H]inositol added at the time of plating. Thirty min before agonist stimulation medium was removed and replaced with Krebs–Ringer–HEPES buffer pH 7.4, containing 10 10 mM glucose and 15 mM LiCl (short term incubations up to 1 h) or with fresh medium (long term studies). Hepatocytes were stimulated with agonists as indicated, and the reaction was stopped by removing buffer (or medium) and adding 1 ml ice-cold 0.4 M perchloric acid. Samples were harvested and neutralized as described above for inositol 1,4,5-trisphosphate assays. The neutralized supernatants were applied on columns containing 1 ml Dowex AG 1-X8 resin. The columns were washed with 20 ml distilled water and 10 ml 5 mM sodium tetraborate/60 mM ammonium formate, and inositol phosphates were eluted with 10 ml 1 M ammonium formate/0.1 M formic acid. Radioactivity in lipids was determined either by dissolving the washed protein pellet in 0.08 M NaOH and counting an aliquot, or by extracting lipids as described above for phosphatidylinositol 4,5-bisphosphate. Extracted lipids were converted to their corresponding glycerophosphoinositols with methylamine, as described above, and separated on Dowex AG 1-X8 columns. Using these labeling conditions, no

agonist-induced changes in labeling of total or individual phosphoinositides were observed (data not shown).

RESULTS

Sustained Accumulation of DAG in Response to Prostaglandin $F_{2\alpha}$, Norepinephrine, Vasopressin, and Angiotensin II

GPCR agonists can induce a prolonged DAG accumulation in the hepatocytes [Dajani et al., 1999]. Figure 1 shows that while NE and prostaglandin $F_{2\alpha}$ induced a sustained, monophasic increase in DAG mass, the accumulation induced by VP and Ang.II was clearly biphasic, with a large initial increase, followed by a lower, sustained level, which may be compatible with partial desensitization (see below). In the experiments below, we have investigated the basis for the prolonged elevation of DAG in response to these agents.

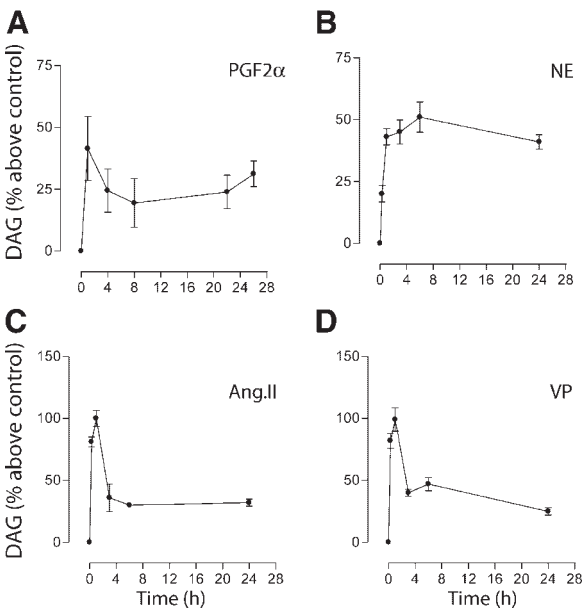

Fig. 1. Time course of agonist-induced DAG accumulation. Hepatocytes were isolated and cultured as described in Materials and Methods. Agonists were added 3 h after plating, using the following concentrations: (**A**) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 100 μM. **B**: norepinephrine (NE), 10 μM. Timolol, 10 μM, was added 15 min prior to NE. **C**: angiotensin II (Ang.II) 1 μM. **D**: vasopressin (VP) 1 μM. The reaction was stopped at the indicated time points, and DAG was extracted and analyzed as described in Materials and Methods. Results are presented as mean % increase above control ±SE of 4–6 experiments. Basal levels of DAG were 2.24 ± 0.09 nmol/mg protein at the time of agonist addition, and increased to 10.23 ± 2.23 nmol/mg protein at 24 h after plating.

Evidence Against a Role of PLD in DAG Generation

Previous studies in hepatocytes have shown that PLD activity induced by various activators is rapidly desensitized [Moehren et al., 1994; Dajani et al., 1999], suggesting that it is unlikely to contribute to sustained DAG accumulation. To further explore the role of PLD in DAG generation, we first compared PLD activation and DAG accumulation induced by GPCR agonists, the protein kinase C activator TPA, and the calcium ionophore A23187, which also activate PLD in the hepatocytes [Bocckino et al., 1987; Moehren et al., 1994]. While TPA and A23187 were far more efficient in stimulating PLD activity than the GPCR agonists (Fig. 2A), stimulation with TPA resulted in only modest DAG generation, and A23187 was not more efficient than VP (Fig. 2C), suggesting that conversion of PA does not constitute a major source of DAG accumulation in response to GPCR agonists. We next stimulated hepatocytes in the presence of 1-butanol, which traps PA as phosphatidylbutanol, and thus prevents its conversion to DAG. 1-butanol dose-dependently suppressed DAG accumulation induced by A23187, while Ang.II-induced DAG production was not significantly affected (Fig. 2B). Figure 2C shows DAG accumulation in response to various stimulators in the presence of 50 mM 1-butanol (using 50 mM 2-butanol as control) to examine the contribution from PLD to DAG accumulation. We found no significant inhibition of DAG production induced by GPCR agonists, while the effects of A23817 and TPA were suppressed (Fig. 2C). Taken together, these results support the conclusion that PLD is unlikely to play a major role in sustained DAG accumulation induced by agonists acting on GPCRs in hepatocytes.

Accumulation of Inositol Phosphates in the Absence of Detectable Increases in $InsP_3$ Mass

Since both NE and prostaglandin $F_{2\alpha}$ induce sustained elevations of DAG in the absence of significant increases in $InsP_3$ mass [Dajani et al., 1999; Sandnes et al., 1999] (and Fig. 1), and apparently not as a result of PLD activity (Fig. 2), we examined whether phosphoinositide breakdown might take place without detectable increases in $InsP_3$. Accumulation of total inositol phosphates in hepatocytes labeled with [^{3}H]inositol was compared to increases in $InsP_3$

mass upon stimulation with various GPCR agonists, in hepatocytes incubated in the presence of lithium (Fig. 3). Both with respect to $InsP_3$ and inositol phosphate accumulation the order of efficacy for agonists was VP $\geq$ Ang.II > NE > prostaglandin $F_{2\alpha}$. VP and Ang.II rapidly increased $InsP_3$ levels, which then declined to a lower sustained level, with a more rapid decrease after Ang.II stimulation, and these responses correlated well with the accumulation of inositol phosphates. $InsP_3$ increase was slight and inconsistent in response to NE, and not detectable after prostaglandin $F_{2\alpha}$, but both these agonists induced significant accumulation of inositol phosphates in all experiments, but with a delayed and more sustained time course compared to responses to VP and Ang.II. These results suggest either that NE and prostaglandin $F_{2\alpha}$ induce a modest breakdown of PIP_2, which is coupled with efficient metabolism of $InsP_3$, or that these agonists stimulate breakdown of other phosphoinositides (i.e. phosphatidylinositol (PI) or phosphatidylinositol 4-phosphate (PIP)), which would generate DAG in the absence of $InsP_3$.

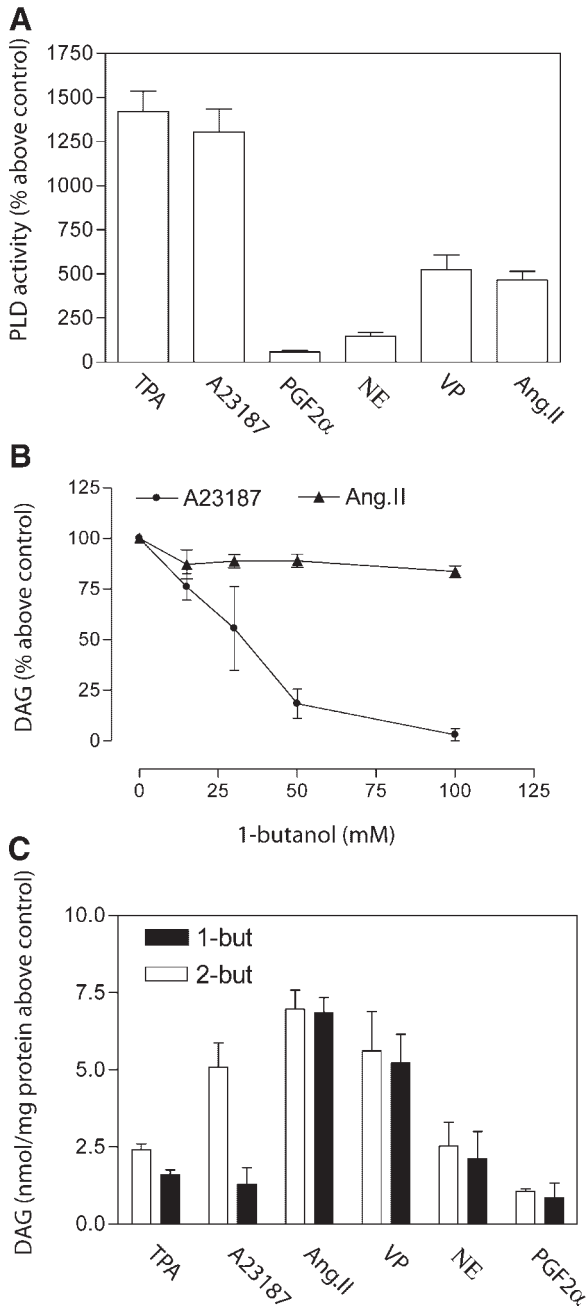

Fig. 2. PLD activity and DAG accumulation induced by GPCR agonists, TPA, and A23187. Hepatocytes were isolated and cultured as described in Materials and Methods. Cells were stimulated 3 h after plating. **A**: PLD activity was determined by incubating hepatocytes in the presence of 30 mM 1-butanol for 15 min. The concentrations used were 1 μM VP, 1 μM Ang.II, 100 μM NE (with 10 μM timolol added 15 min earlier), 100 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 1 μM TPA, and 1 μM A23187. Phosphatidylbutanol was extracted and analyzed as described in Materials and Methods. The control level of phosphatidylbutanol was 3169 ± 683 dpm/mg protein. Results are presented as mean % increase above control ±SE (n = 5). **B**: Effect of increasing concentrations of 1-butanol on DAG accumulation induced by 1 μM A23187 or 1 μM Ang.II.1-Butanol was added 5 min prior to agonists, and cells were stimulated for 15 min with agonists. The reaction was stopped and DAG was extracted and analyzed as described in Materials and Methods. Results are presented as mean % increase above control ±SE of five experiments each, where the increase in the absence of 1-butanol was set at 100%. The increase in DAG above control induced by A23187 in the absence of 1-butanol was 4.60 ± 0.75 nmol/mg protein, and the increase induced by Ang.II was 5.13 ± 1.13 nmol/mg protein. **C**: Effect of 50 mM 1-butanol or 2-butanol on agonist-induced DAG accumulation. The same concentrations of agonists as in A were used. Butanol was added 5 min prior to agonists, and cells were stimulated with agonists for 15 min. Results are presented as mean increase ±SE (n = 4–6) in DAG above control. Control levels of DAG were 2.34 ± 0.23 nmol/mg protein (n = 6).

GPCR agonists Induce Sustained Activation of Phospholipase C

To further examine the role of PLC in sustained DAG accumulation, we studied the duration of inositol phosphate accumulation after stimulation of hepatocytes with the GPCR agonists. Agonists were added to cultures 3 h after plating, and then inositol phosphates were collected, in the presence of lithium, during 30 min periods at various times after addition of agonists. The results showed that both basal and agonist-stimulated inositol phosphate accumulation decreased with time in culture (Fig. 4), presumably due to a gradual decline in PLC activity upon culturing of hepatocytes.

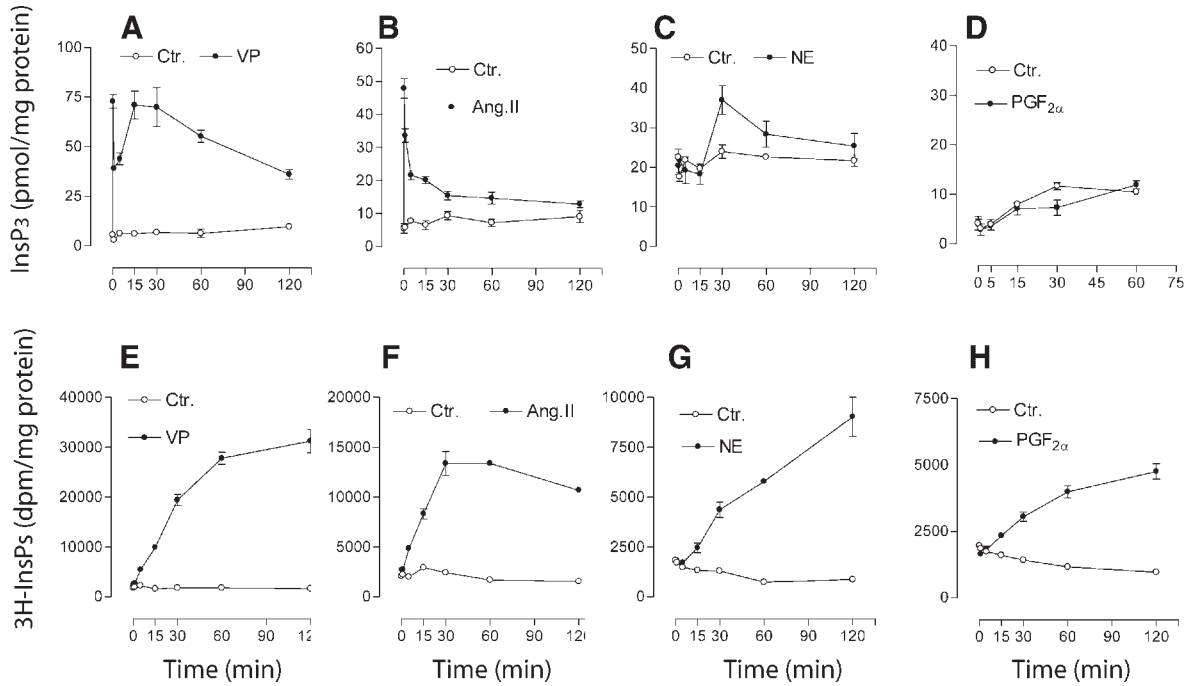

Fig. 3. Time course of agonist-induced $InsP_3$ and inositol phosphate accumulation. Hepatocytes were isolated and cultured for 3 h as described in Materials and Methods. Medium was removed and replaced with Krebs–Ringer HEPES buffer, pH 7.4, containing 10 mM glucose and 15 mM LiCl. Cells were preincubated for 15 min before addition of agonists: VP, 1 μM, Ang.II, 1 μM, NE, 100 μM, with 10 μM timolol added 15 min earlier, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 100 μM. The reaction was stopped at the indicated time points by removing the buffer and adding 0.4 M ice-cold perchloric acid. $InsP_3$ (**A**–**D**) or inositol phosphates (**E**–**H**) were extracted and analyzed as described in Materials and Methods. Results are presented as mean ± SE of three replicates. Typical results of single experiments, with $InsP_3$ and inositol phosphates determined in parallel with each agonist are shown. The experiments were repeated at least four times with similar results.

There was a time-dependent, agonist-specific decrease in stimulated inositol phosphate accumulation, which was not due to metabolism of agonists (data not shown). Responses to VP and Ang.II were significantly attenuated within 1 h after addition, whereas responses induced by NE and prostaglandin $F_{2\alpha}$ were more sustained. To more directly assess the extent of desensitization, hepatocytes were pretreated with VP or NE for 6 h, followed by washing and restimulation with fresh agonist. Inositol phosphate accumulation in response to VP was strongly attenuated, while the response to NE showed little desensitization (Fig. 4). Taken together, the results are compatible with NE inducing a more sustained DAG accumulation than VP and Ang.II because of less desensitization (cf Fig. 1).

While the above results suggested that phosphoinositide breakdown might account for the sustained accumulation of DAG induced by GPCR agonists, they did not show whether PIP_2 is the only source of DAG. Neomycin, which binds with high affinity to PIP_2, has been widely used to assess the role of PIP_2 breakdown in various cells [Schacht, 1976; Hildebrandt et al., 1997]. However, neomycin at concentrations up to 1 mM did not affect VP-induced IP_3 accumulation in hepatocytes (data not shown). We therefore used wortmannin, which has been found to inhibit PI 4-kinase activity, resulting in inhibition of PIP, and consequently PIP_2 synthesis [Nakanishi et al., 1995; Balla et al., 1997; Meyers and Cantley, 1997]. Pretreatment of hepatocytes with wortmannin almost completely prevented inositol phosphate accumulation as well as DAG accumulation induced by the GPCR agonists (Fig. 5). DAG accumulation in response to TPA and A23187 was blunted (Fig. 5B), presumably due to the decrease in PIP_2 mass (see Fig. 7), as PIP_2 is required for the catalytic activity of PLD [Hammond et al., 1995; Exton, 2002]. Furthermore, addition of wortmannin to hepatocyte cultures that had been stimulated with agonists for 3 h in the absence of lithium almost completely prevented further inositol phosphate accumulation when lithium was added (data not shown). While these results suggest that neither the initial nor the sus-

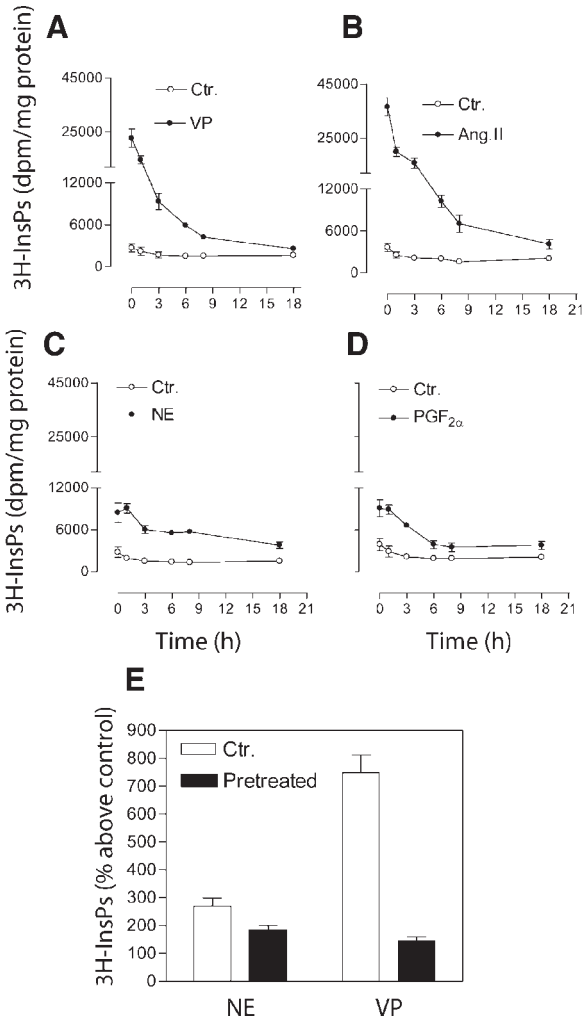

Fig. 4. Duration of agonist-induced inositol phosphate accumulation. Hepatocytes were isolated and cultured for 3 h in the presence of 5 μCi/ml [2-^{3}H]inositol as described in Materials and Methods. Medium was replaced with fresh medium without radiolabeled inositol, and after 15 min preincubation, agonists were added: (**A**) 1 μM VP, (**B**) 1 μM Ang.II, (**C**) 100 μM NE (with 10 μM timolol added 15 min earlier) (NE), (**D**) or 100 μM prostaglandin F_2 ($PGF_{2\alpha}$). 15 mM LiCl was added at the times indicated, and inositol phosphates were allowed to accumulate for 30 min. The reaction was stopped and inositol phosphates were collected and analyzed as described in Materials and Methods. Results are presented as mean ± SE of four experiments. **E**: Hepatocytes were cultured as described above and incubated with saline, 1 μM VP or 100 μM NE (with 10 μM timolol added 15 min earlier) for 6 h before cells were washed twice with medium, followed by addition of fresh medium containing 15 mM LiCl. Cells were preincubated for 15 min, before stimulation with 1 μM VP or 100 μM NE (in the presence of 10 μM timolol) for 30 min. Results are presented as % increase in inositol phosphates above control and represent mean ± SE of four experiments. Control levels of inositol phosphates were 3,085 ± 496 dpm/mg protein.

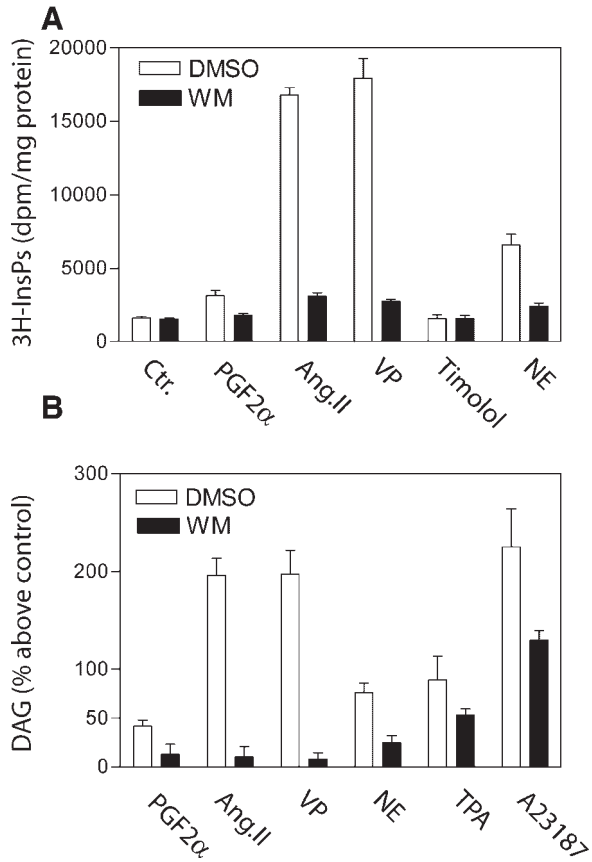

Fig. 5. Effect of wortmannin on inositol phosphate and DAG accumulation. Hepatocytes were isolated and cultured for 3 h as described in Materials and Methods. **A**: Medium was replaced with Krebs–Ringer HEPES buffer, pH 7.4, containing 10 mM glucose and 15 mM LiCl. Wortmannin (10 μM) or the vehicle DMSO was added simultaneously. After 15 min preincubation, hepatocytes were stimulated with 1 μM VP, 1 μM Ang.II, 100 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), or 100 μM NE (with 10 μM timolol added 15 min earlier) (NE). Inositol phosphates were collected after 30 min of stimulation and analyzed as described in Materials and Methods. Results are presented as mean ± SE of four experiments. **B**: Wortmannin (WM) (10 μM) or the vehicle DMSO was added to the medium, and cells were preincubated for 15 min before agonists were added as described above. The reaction was stopped after 15 min, and DAG was extracted and analyzed as described in Materials and Methods. Results are presented as mean % increase ±SE (n = 4) in DAG above control. Control levels of DAG were 2.40 ± 0.07 nmol/mg protein in DMSO-treated cells and 2.17 ± 0.28 nmol/mg protein in wortmannin-treated cells.

tained DAG accumulation results from PI hydrolysis, they do not exclude the possibility that PIP breakdown might contribute.

Quantitative Relationship Between Changes in $InsP_3$, PIP_2, and DAG Mass

To further examine the role of PIP_2 breakdown as a source of sustained DAG generation we performed a detailed analysis of changes in

PIP_2, $InsP_3$, and DAG mass upon agonist stimulation of hepatocytes. In these experiments we used VP, which induces robust increases in $InsP_3$ and DAG mass. Both DAG and $InsP_3$ mass reached peak levels 15 min after addition of VP and then slowly declined (Fig. 6A,B). (The slower time course of $InsP_3$ elevation in these experiments, compared to Fig. 3 is due to the lower concentration of VP used here). PIP_2 mass reached a nadir 5 min after addition of agonist, and then gradually increased towards control level (Fig. 6C), while $InsP_3$ levels remained elevated. Because metabolism of $InsP_3$ is rapid (see below), this suggests that efficient resynthesis of PIP_2 allows a continued production of $InsP_3$ and that the decline in $InsP_3$ production at later time points may be due to desensitization, rather than substrate depletion.

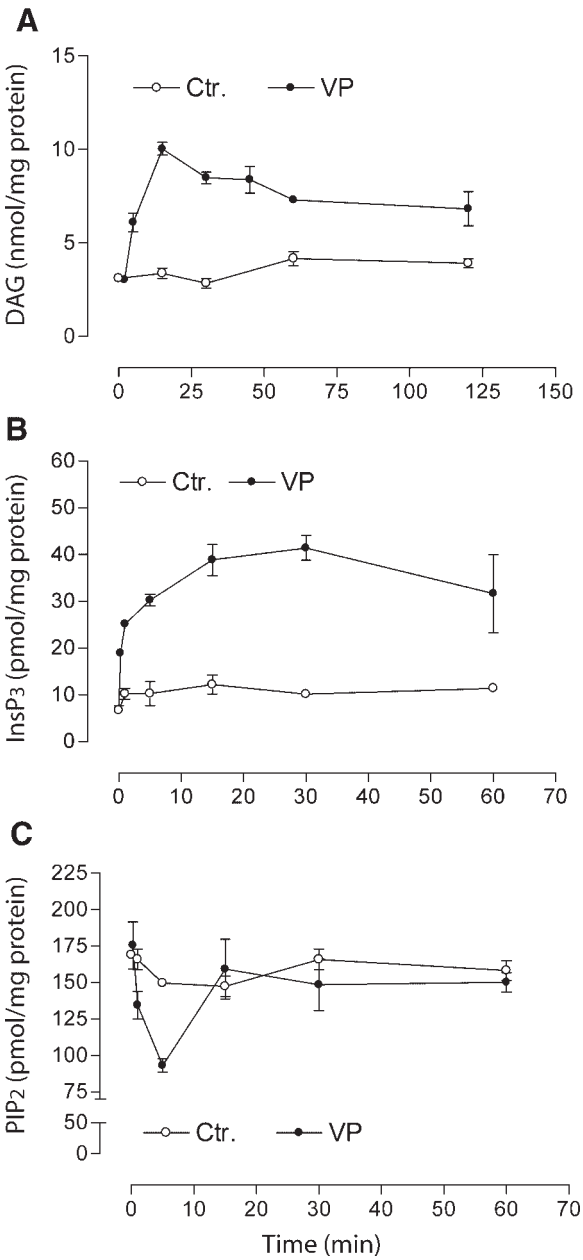

Fig. 6. Time course of VP-induced changes in the level of DAG, $InsP_3$, and PIP_2. Hepatocytes were cultured for 3 h before stimulation with 10 nM VP. The reaction was stopped at the indicated time points, and (**A**) DAG, (**B**) $InsP_3$, and (**C**) PIP_2 were extracted and analyzed as described in Materials and Methods. Results from one typical experiment are shown. The experiment was repeated five times with similar results.

Further support for the idea that resynthesis of PIP_2 is highly efficient was obtained by treating hepatocytes with wortmannin before stimulation with VP. This pretreatment strongly reduced the initial phase of VP-stimulated $InsP_3$ accumulation and abolished the sustained phase (Fig. 7A,B). Furthermore, while PIP_2 mass in the hepatocytes was reduced by wortmannin, VP induced a further decrease, and resynthesis was prevented in the presence of wortmannin (Fig. 7C,D). In wortmannin-treated cells, the VP-induced decrease in PIP_2 mass during the first minute of stimulation was 10-fold higher than the increase in $InsP_3$ mass in the same period (57.6 ± 21.4 vs 6.0 ± 1.8 pmol/mg protein, $n = 5$), indicating rapid metabolism of $InsP_3$.

The above results suggested that $InsP_3$ is rapidly metabolized in the hepatocytes. If DAG accumulation resulted from PIP_2 breakdown only, the differences between increases in DAG and $InsP_3$ mass following agonist stimulation (approximately 100-fold, Fig. 6A,B) could be a result of greatly differing rates of metabolism. We therefore used a VP receptor antagonist ([des-Gly^9-β-mercapto-β,β-cyclopentamethylene-propionyl^1 O-Et-Tyr^2,Val^4, Arg^8]-VP) to inhibit further activation of PLC in order to estimate the half-lives of DAG and $InsP_3$. The experiments showed that breakdown of $InsP_3$ was rapid (Fig. 8A), with an average half-life of 0.6 ± 0.1 min ($n = 4$). Similarly, PIP_2 resynthesis in the presence of the antagonist was rapid (Fig. 8B). In contrast, disappearance of DAG was slower, with an average half-life of 10.6 ± 1.5 min ($n = 4$) (Fig. 8C). Thus, the half-life of DAG was approximately 20-fold longer than the half-life of $InsP_3$.

DISCUSSION

The main findings of the present study is that phosphoinositide turnover, like DAG accumu-

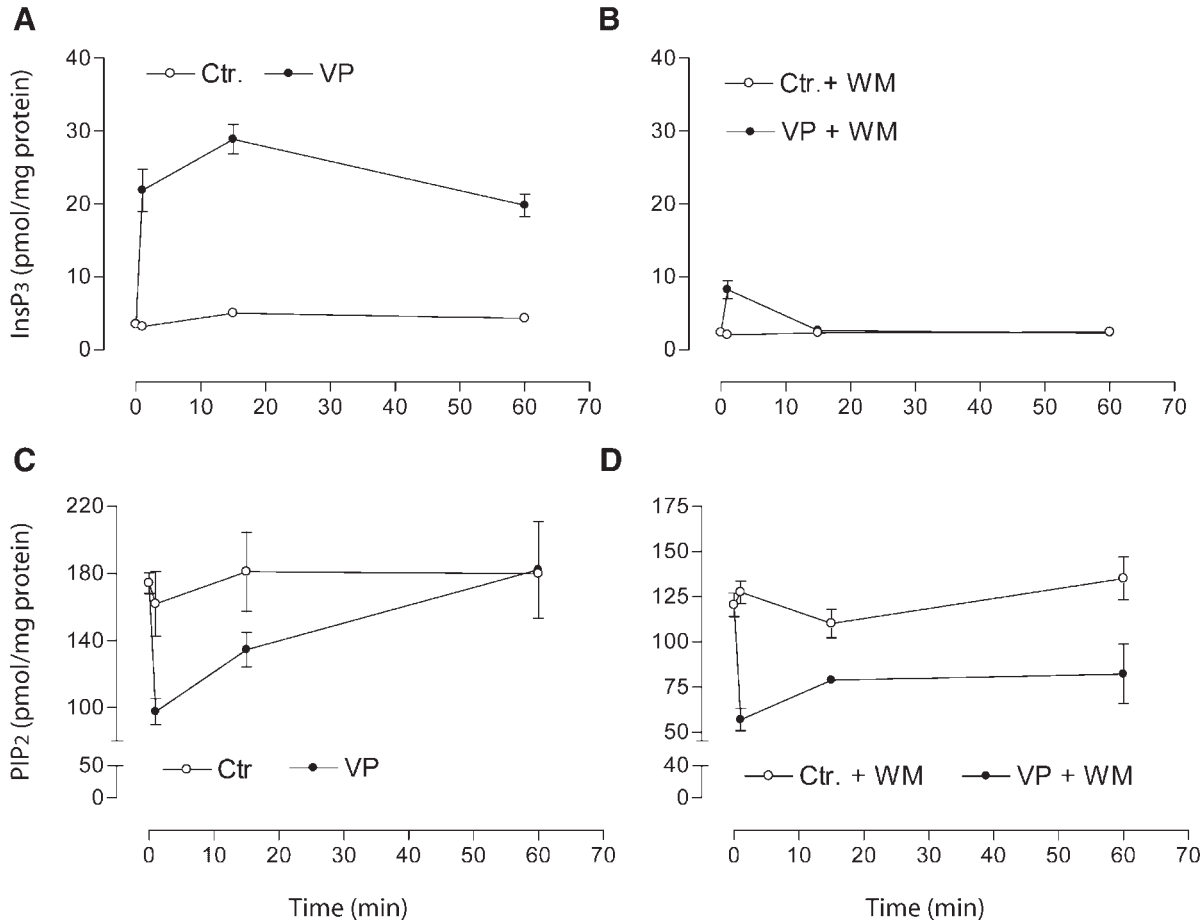

Fig. 7. Effect of wortmannin pretreatment on VP-induced changes in the level of $InsP_3$ and PIP_2. Hepatocytes were cultured for 3 h. Medium was replaced with Krebs–Ringer HEPES buffer, pH 7.4, containing 10 mM glucose. Cells were pretreated for 15 min with the vehicle DMSO (**A**, **C**) or 10 μM wortmannin (WM) (**B**, **D**) before stimulation with 1 μM VP. The reaction was stopped at the indicated time points, and $InsP_3$ (A, B) and PIP_2 (C, D) were extracted and analyzed as described in Materials and Methods. Results from one typical experiment, presented as mean ± SE of three replicates are shown. The experiment was repeated five times with similar results.

lation, induced by activation of GPCRs in hepatocyte cultures is sustained for at least 18 h, and that DAG is metabolized at a much slower rate than $InsP_3$, suggesting that phosphoinositide turnover may account for a major part of the DAG generated during prolonged agonist stimulation. This is in contrast to the prevailing view that although transient increases in DAG mass can be ascribed to phosphoinositide breakdown, sustained DAG accumulation results from PC breakdown [Exton, 1990; Nishizuka, 1995; Quest et al., 1996].

The idea that agonist-stimulated DAG accumulation, and, in particular, sustained elevation, depends on PC breakdown, has gained support from the finding of water-soluble choline metabolites [Slivka et al., 1988; Cook and Wakelam, 1989; Plevin et al., 1991; Cook and Wakelam, 1992], the temporal dissociation between generation of $InsP_3$ or inositol phosphates and DAG accumulation [Wright et al., 1988; Cook et al., 1990], and fatty acid analysis of DAG, PC, and PI (reviewed in [Exton, 2002]). The present results suggest that PC hydrolysis, mediated by PLD, is unlikely to account for the sustained DAG generation induced by GPCR agonists in hepatocytes. Rapid desensitization of PLD activity stimulated by GPCR agonists appears to be a general feature [Cook et al., 1991; McKenzie et al., 1992; Plevin and Wakelam, 1992; Nieto et al., 1994; Schmidt et al., 1995], and previous studies have shown that PLD activity induced by GPCR agonists, TPA, and A23187 in hepatocytes is rapidly desensitized [Moehren et al., 1994; Dajani et al., 1999]. We therefore examined the role of PLD in the

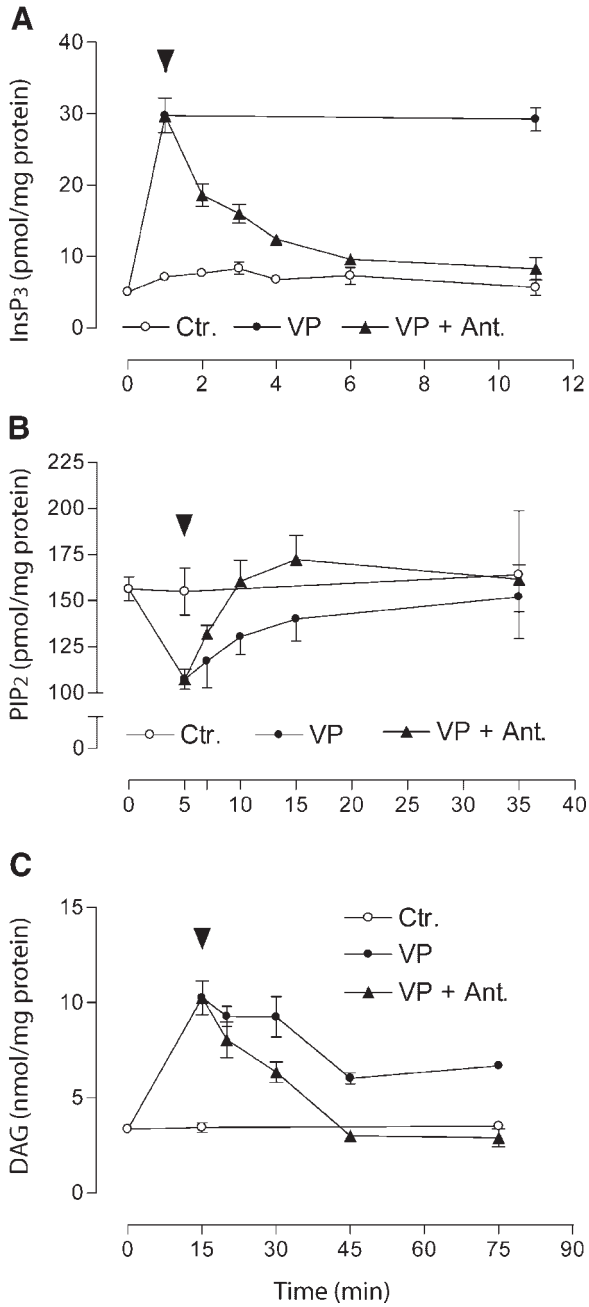

Fig. 8. Degradation of $InsP_3$ and DAG, and resynthesis of PIP_2 upon blockade of VP receptors. Hepatocytes were cultured for 3 h before stimulation with 10 nM VP. At the times indicated, 1 μM of the VP receptor antagonist [des-Gly^9-β-mercapto-β, β-cyclopentamethylenepropionyl^1 O-Et-Tyr^2,Val^4,Arg^8]-VP was added (VP + Ant.), and the reaction was stopped as indicated. $InsP_3$ (**A**), PIP_2 (**B**), and DAG (**C**) were extracted and analyzed as described in Materials and Methods. Results from single experiments, presented as mean ± SE of three replicates are shown. The experiments were repeated at least four times with similar results.

initial DAG accumulation induced by GPCR agonists. In agreement with previous studies in freshly isolated hepatocytes [Allan and Exton, 1993; Moehren et al., 1994], we found that inhibition of conversion of PA to DAG with 1-butanol did not significantly affect DAG generation induced by GPCR agonists, while the effect of TPA was partly suppressed and the effect of A23187 was strongly inhibited. These findings suggest that Ca^{2+}-mobilization and protein kinase C activation induced by agonists are insufficient to elicit DAG formation via PLD activity in hepatocytes. Another possibility is that PC breakdown is mediated by a PC-PLC activity [Irving and Exton, 1987; Augert et al., 1989a,b; Exton, 1990]. However, the presence of PC-PLC in mammalian cells has not been firmly established, and we have not found any evidence of sustained PC breakdown in choline-labeled hepatocytes (data not shown). Finally, sphingomyelin synthase activity might contribute to DAG accumulation [Luberto and Hannun, 1998]. The latter activity generates sphingomyelin from PC and ceramide. Ceramide may be determined simultaneously with DAG in the assay used here [Preiss et al., 1987], however, we have found no evidence of altered ceramide levels in hepatocytes during sustained DAG accumulation (data not shown).

In contrast to the lack of evidence of PC hydrolysis as a source of DAG generation in the hepatocytes, the finding that phosphoinositide breakdown was sustained for up to 18 h, although partially desensitized, raises the possibility that DAG may originate from phosphoinositides also during long-term responses to GPCR agonists. It has previously been reported that phosphoinositide breakdown can account for most of the DAG and PA generated in freshly isolated hepatocytes during 1 h of stimulation with VP [Allan and Exton, 1993]. Our data suggest that even a much longer DAG elevation may result from phosphoinositide hydrolysis. It has been argued that, because of limited supply, phosphoinositide breakdown cannot account for the amount of DAG accumulated upon agonist stimulation [Augert et al., 1989a; Exton, 1997]. However, agonists that activate PLC also stimulate resynthesis of phosphoinositides [Downes et al., 1989; Stephens et al., 1993; Cunningham et al., 1995; Nakanishi et al., 1995; Huang et al., 2002], possibly through mechanisms involving low-molecular weight GTPases [Stephens et al., 1993; Tolias et al.,

1995; Weernink et al., 2000], PA [Jenkins et al., 1994; Honda et al., 1999], and phosphatidylinositol transfer protein [Cockcroft, 1998]. It was recently shown that resynthesis of PIP_2 was highly efficient and not limiting for agonist-induced responses in a neuroblastoma cell line [Willars et al., 1998b]. We show here that PIP_2 levels are only transiently decreased, while the increase in $InsP_3$ mass is sustained. Furthermore, metabolism of $InsP_3$ is swift, indicating that rapid resynthesis of PIP_2 supports sustained phosphoinositide hydrolysis also in the hepatocytes. Assuming no metabolism of DAG during the initial 15 min of VP stimulation (Fig. 6), the average rate of synthesis of DAG in the hepatocytes over this period was calculated to be 376 ± 44 pmol/mg protein/min ($n = 8$), which is a minimum value and probably an underestimate, since some breakdown is likely to occur. The average level of PIP_2 was 175.2 ± 14.5 pmol/mg protein ($n = 12$). To sustain the DAG production during the initial 20–30 min of VP stimulation would thus require turnover of the total pool of PIP_2 approximately every 30 s, which is in agreement with previous measurements in freshly isolated hepatocytes, based on radiolabeling [Creba et al., 1983; Thomas et al., 1984; Palmer et al., 1986; Downes, 1989]. In contrast, the rate of synthesis of $InsP_3$ (assuming no breakdown in this period) even during the initial 15 s of stimulation was only 62.8 ± 13.6 pmol/mg/min ($n = 4$). However, the short half-life of $InsP_3$, the strongly blunted $InsP_3$ response in experiments using wortmannin to inhibit resynthesis of PIP and PIP_2 (Fig. 7), and the accumulation of inositol phosphates in the absence of significant increases in $InsP_3$ mass (Fig. 3), together suggest that changes in the level of $InsP_3$ and PIP_2 do not fully reflect the magnitude of polyphosphoinositide turnover. Furthermore, the half-life of DAG was at least 20-fold higher than the half-life of $InsP_3$. This difference is probably an underestimate, as the half-life of $InsP_3$ has been estimated to be less than 10 seconds in radiolabeling studies [Hughes and Putney, 1989; Fisher et al., 1990].

It is presently not clear whether PIP_2 is the only substrate for the PLC activity that generates DAG. Studies in various cell types showing agonist-induced, transient increases in $InsP_3$, despite sustained accumulation of inositol phosphates [Tilly et al., 1988; Chilvers et al., 1991; Murthy and Makhlouf, 1991; Plevin and Wakelam, 1992], have been interpreted to suggest that PI or PIP was the major substrate for PLC during prolonged inositol phosphate generation [Wilson et al., 1985; Imai and Gershengorn, 1986; Ackermann et al., 1987; Murthy and Makhlouf, 1991; Biden et al., 1992; Button et al., 1994; Woodcock, 1995; Mitchell et al., 2001], while other studies have not found inositol phosphate generation from PI or PIP [Hughes and Putney, 1989; Fisher et al., 1990]. Experiments using wortmannin to inhibit PIP and PIP_2 synthesis did not indicate PI breakdown. However, wortmannin also inhibits PI 3-kinase, which in some cells may activate PLCγ [Bae et al., 1998; Falasca et al., 1998], and it was recently found that carbachol stimulates PI hydrolysis by PI 3-kinase-dependent stimulation of PLCγ in pancreatic islets [Mitchell et al., 2001]. We can presently not exclude that this might also occur in hepatocytes, and that hydrolysis of several phosphoinositides contributes to sustained DAG accumulation.

We found that responses to GPCR agonists desensitized in an agonist-specific manner. Inositol phosphate accumulation in response to Ang.II and VP desensitized rapidly, which may account for the biphasic accumulation of DAG. In contrast, responses to prostaglandin $F_{2\alpha}$ and NE showed little desensitization during the first hours of stimulation, consistent with the sustained, monophasic accumulation of DAG. The different patterns of desensitization may be related to differential trafficking of phosphorylated receptors [Fonseca et al., 1995; Oakley et al., 2001]. In addition, the different agonist profiles may reflect differences in activation of PLC isoforms. In particular, the delayed or absent accumulation of IP_3 in the presence of inositol phosphate accumulation induced by NE and prostaglandin $F_{2\alpha}$ might be due to preferential activation of PLCγ-mediated hydrolysis of PI, which may be less sensitive to desensitization [Biden et al., 1993].

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