Quantification of inositol phospholipid breakdown in isolated rat hepatocytes

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The hydrolysis of inositol phospholipids induced by vasopressin in hepatocytes during 60 min was quantified chemically. There was a large release of *myo*-inositol which was abolished by Li^+ , indicating that it was derived from inositol phosphates and not from phospholipase D action on PtdIns. There was also a large release of inositol phosphates which was increased approx. 2-fold by Li^+ at 30 min, but then remained constant, suggesting that inositol phospholipid breakdown declined substantially beyond this time. In cells prelabelled with *myo*- ^3H inositol and treated with Li^+ , ^3H PtdIns(4,5) P_2 decreased maximally (50%) at 15 s and then recovered to a level at 5 min that was maintained at 25% below control for 40 min. ^3H PtdIns4P and ^3H PtdIns showed slower decreases to approx. 30% below control at 15 min, but with no further changes. Labelled Ins(1,4,5) P_3 and Ins(1,3,4) P_3 showed 2–4-fold increases within 30 s and then declined to values that were maintained at a constant level above the control, except for ^3H Ins(1,3,4) P_3 , which showed a second increase. ^3H Ins(1,4) P_2 showed a very large increase over 10 min, whereas ^3H Ins4P and ^3H Ins1P showed little change before 6 and 15 min respectively. The total ^3H inositol phosphates showed little further increase after 20 min. These data are

consistent with a rapid, but not sustained, hydrolysis of PtdIns(4,5) P_2 , but not of PtdIns, by phospholipase C, but do not exclude PtdIns4P as a substrate. Phosphatidate was rapidly increased by vasopressin, whereas diacylglycerol was increased after a 1–2 min lag. Both were maintained at levels 2–3-fold above control for 60 min. The vasopressin-induced increase in inositol phosphates plus *myo*-inositol (approx. 120 nmol/100 mg) was greater than the increase in diacylglycerol plus phosphatidate (approx. 60 nmol/100 mg) between 10 and 40 min. This indicates that there was substantial further metabolism of these lipids. Addition of 75 mM ethanol resulted in rapid production of phosphatidylethanol in response to vasopressin and a 35% reduction in phosphatidate, but no decrease in diacylglycerol. In summary, the results indicate that inositol phospholipid hydrolysis by phospholipase C can account for most of the diacylglycerol and phosphatidate that accumulate during 60 min of vasopressin action, but that these phospholipids are probably not the major source of the phosphatidate that is formed during the first 2 min by phospholipase D, or of the diacylglycerol and phosphatidate that are formed beyond 30 min.

INTRODUCTION

It is well established that certain hormones, growth factors and neurotransmitters stimulate the hydrolysis of PtdIns(4,5) P_2 by phospholipase C (PLC) to generate Ins(1,4,5) P_3 and 1,2-diacylglycerol (DAG), which cause intracellular Ca^{2+} mobilization and protein kinase C activation respectively. The receptor–PLC coupling systems involve pertussis toxin-sensitive or -insensitive G proteins, or growth factor receptor tyrosine kinases [1]. It is also known that choline and phosphocholine are formed when many cells and tissues are stimulated with agonists [2,3] and that this is due to activation of phospholipases acting on phosphatidylcholine (PtdCho).

Previous studies from our laboratory have focused on short-term (e.g. 10 min) DAG and phosphatidic acid (PtdOH) accumulation and either inositol phospholipid or PtdCho hydrolysis in rat hepatocytes stimulated by Ca^{2+} -mobilizing hormones [4–7]. The decreases in PtdIns, PtdIns4P and PtdIns(4,5) P_2 were quantified [6] and the results supported an earlier conclusion that these phospholipids were not the only source of DAG [4]. By using hepatocytes prelabelled with ^3H alkyl-lysoglycerophosphocholine or by analysing the molecular species of DAG, it was concluded that PtdCho was a contributor to DAG accumulation in vasopressin-stimulated hepatocytes [7]. However, these data did not indicate the quantitative magnitude of the contribution. It was also discovered that PtdOH accumulated more rapidly than DAG in stimulated hepatocytes [5] and that

phosphatidylethanol (PtdEtOH) was rapidly formed in the presence of ethanol [8]. These observations led to the conclusion that activation of phospholipase D (PLD) was a rapid response to Ca^{2+} -mobilizing agonists [5], although PtdCho was not shown to be the substrate.

Most studies of the breakdown of inositol phospholipids in cells have utilized isotopic labelling of lipid precursors and analysis of labelled metabolites, and there have been few investigations employing chemical measurements. In addition, most studies have examined the changes occurring during the first 20 or 30 min of agonist stimulation, but not later changes. The present study was initiated to determine chemically the magnitude and the time course of inositol phospholipid hydrolysis in hepatocytes stimulated with vasopressin for 60 min. The following questions were asked. (1) How do the previously reported quantitative decreases in the levels of PtdIns and PtdIns P_2 correspond to the changes in free *myo*-inositol and inositol phosphates? (2) Is there evidence that PtdIns is a direct substrate for PLC or PLD? (3) How prolonged is the accumulation of DAG and PtdOH, and how does its magnitude compare with that of inositol phospholipid breakdown? To answer these questions, we quantified the chemical changes in free *myo*-inositol, inositol phosphates, PtdOH and DAG induced by vasopressin, and analysed the radioactivity in individual inositol phosphates and inositol phospholipids in hepatocytes previously labelled with *myo*- ^3H inositol. The data indicate that PtdIns(4,5) P_2 degradation in response to vasopressin occurs to a much

Abbreviations used: PtdCho, phosphatidylcholine; PtdEtOH, phosphatidylethanol; PtdOH, phosphatidate; DAG, 1,2-diacylglycerol; InsPs, inositol phosphates; PLD, phospholipase D; PLC, phospholipase C.

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greater extent than previously surmised, and that there is little or no direct hydrolysis of PtdIns by PLC or PLD.

EXPERIMENTAL

Materials

Unless otherwise indicated, reagents were from Sigma and organic solvents (Optima grade) were from Fisher. *myo*-[2-³H]inositol (17 Ci/mmol) was from Amersham. Pyridine, *NO*-bis-(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane were from Pierce. Alkaline phosphatase (calf intestine) was from Boehringer Mannheim and *chiro*-inositol was a gift from Dr. William Sherman, Washington University, St. Louis, MO, U.S.A.

Isolation and incubation of hepatocytes

Hepatocytes prepared [9] from fed male Sprague-Dawley rats (250–300 g) were used at > 92% viability as assessed with Trypan blue. They were suspended at 60–70 mg wet wt./ml in Krebs-Henseleit bicarbonate buffer supplemented with 1.5% gelatin and gassed with 95% O₂/5% CO₂ at 37 °C.

Measurement of *myo*-inositol and inositol phosphates by gas chromatography

To assess accumulation of inositol phosphates and free *myo*-inositol, cells were pretreated for 15 min with or without 15 mM LiCl before incubation with 0.9% saline/0.1% albumin or 100 nM vasopressin. Samples (1 ml) were extracted with 2 ml of 15% trichloroacetic acid for 20 min on ice. After centrifugation (2000 g, 5 min), the supernatant was removed and 50 nmol of *chiro*-inositol was added as internal standard. The extracts were washed with 3 ml of water-saturated diethyl ether and neutralized to pH 7.5 with 1 M NaHCO₃. The samples were applied to Rexyn ion-exchange resin (1 ml bed volume; Fisher) and eluted with 3 ml of water.

Free *myo*-inositol was derivatized as described below and inositol phosphates were converted to free *myo*-inositol [10] by incubation with 200 µl of 20 mM ethanolamine, 5 mM MgCl₂, pH 8.5, and alkaline phosphatase (350 units/ml) for 2 h at 37 °C. After boiling for 20 min, 10 µl of 0.1 M HCl and 1 ml of water were added and the samples were applied to Rexyn (0.8 ml bed volume) and eluted with 3 ml of water. The degree of conversion of inositol phosphates to free *myo*-inositol was routinely > 90%.

Derivatization of *myo*-inositol was adapted from the method of Leavitt and Sherman [11]. Silylation was achieved using 125 µl of pyridine/*NO*-bis-(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (6:2:1, by vol.). The tightly capped tubes were placed in a container with Drierite desiccant and allowed to derivatize overnight. Gas chromatography utilized a 15 m × 0.25 mm (internal diam.) fused silica capillary column (SPB-1; Supelco) with a Varian 3500 flame ionization detector, connected to a Varian DS654 computer/integrator. A temperature program was developed from 160 to 200 °C after an initial hold of 2 min, followed by a 5 °C/min increase to 200 °C, with a 2 min hold at 200 °C. A 1 µl sample was injected at a 4:1 split ratio. The injector was held at 250 °C and the detector at 260 °C. *chiro*-Inositol was routinely detected at 4.1 ± 0.2 min and *myo*-inositol at 6.3 ± 0.1 min. Free *myo*-inositol and inositol phosphates were quantified by integration with respect to the *chiro*-inositol standard. Values for inositol phosphates were corrected for free *myo*-inositol present before phosphatase treatment.

Analysis of [³H]inositol phospholipids

Hepatocytes were labelled with 0.1 mM *myo*-[2-³H]inositol (10 µCi/ml) for 90 min and then washed and resuspended in medium containing 0.1 mM *myo*-inositol. After preincubation with 15 mM LiCl for 15 min, the cells were treated with saline or vasopressin, and 1 ml aliquots were removed into 2 ml of 15% trichloroacetic acid at the desired times. The supernatant was analysed for inositol phosphates by h.p.l.c. as described below, and the pellet for inositol phospholipids.

After removal of acid-soluble material, the cell precipitate was washed with 1 ml of 5% trichloroacetic acid/1 mM EDTA and 1 ml of water [12]. The pellet was extracted twice with 1.5 ml of chloroform/methanol/12 M HCl (100:100:1, by vol.) at room temperature for 30 min, using centrifugation at 2000 g (5 min), and once with 1.5 ml of chloroform/methanol/12 M HCl (200:100:1, by vol.). The three supernatants were pooled and 1.5 ml of chloroform was added with 1.5 ml of 0.1 M HCl to give chloroform/methanol/HCl (8:4:3, by vol.). After centrifugation (3000 g, 10 min) the lower phase was washed once with 3 ml of chloroform/methanol/0.1 M HCl (3:48:47, by vol.) and dried under N₂. Lipid deacylation was carried out as described previously [13]. The resulting glycerophosphoryl esters were separated using 1 ml of Dowex AG-1X8 resin (100–200 mesh, formate form; Bio-Rad). [³H]Glycero-PtdIns was eluted with 2 × 5 ml of 60 mM ammonium formate/5 mM disodium tetraborate; [³H]glycero-PtdIns4P was eluted with 2 × 5 ml of 400 mM ammonium formate/100 mM formic acid; and [³H]-glyceroPtdIns(4,5)P₂ was eluted with 2 × 5 ml of 1 M ammonium formate/100 mM formic acid. Fractions were counted for radioactivity in 10 ml of ReadySafe (Beckman). A ³H quench curve showed no significant difference between the efficiencies of counting of inositol phospholipids and inositol phosphates.

Measurement of [³H]inositol phosphates by h.p.l.c.

The trichloroacetic acid supernatant, containing inositol phosphate isomers, was removed and dried under vacuum (Speedvac; Savant). Dried extracts were resuspended in 100 µl of water and frozen at –20 °C before h.p.l.c. analysis. Separation of inositol phosphate isomers was achieved using a Whatman Partisil SAX analytical column, with a Solutecon precolumn and pellicular anion-exchange guard column. Sequential gradients of ammonium formate (2 M; pH 3.75 with orthophosphoric acid) were developed [14,15]. The flow rate was 1 ml/min and 1 ml fractions were diluted with 2 ml of 50% (v/v) methanol and counted for radioactivity. The retention times of [³H]inositol phosphate standards, obtained from New England Nuclear, were used to identify inositol phosphates in the samples.

T.l.c. of lipids

PtdOH, PtdEtOH and DAG were quantified by t.l.c. as previously described [5,8], with slight modification. A 1 ml sample of cell suspension was extracted into 1.5 ml of chloroform/methanol (1:2, v/v) [16], and then 0.5 ml of chloroform and 0.5 ml of 0.1 M HCl were added, with vigorous mixing. After centrifugation (2000 g for 15 min), the aqueous layer was discarded and the chloroform layer was filtered through glass wool and dried under N₂.

PtdOH and PtdEtOH were separated from other lipids in a one-dimensional system on 20 cm × 10 cm silica gel 60 F-254 plates (Merck). Samples (equivalent to 5–10 mg of hepatocytes) were applied in 50 µl of chloroform. The plates were developed in ethyl acetate/iso-octane/acetic acid (9:5:2, by vol.) to the top of the plate. After thorough drying, they were dipped in charring

reagent (5% $\text{CuSO}_4/4\%$ H_3PO_4) and heated at 190°C for 15 min. The plates were scanned with an LKB 2202 Ultroscan laser densitometer using dipalmitoyl-PtdOH (Avanti) as standard. Neutral lipids were also separated on the silica gel plates using the solvent system toluene/diethyl ether/ethanol/ammonium hydroxide (50:40:2:0.2, by vol.). Plates were stained with Coomassie Brilliant Blue R-250 (0.03% in 30% methanol/100 mM NaCl) for 30 min and destained for 15 min in 30% methanol [17]. Dried plates were scanned with the LKB 2202 Ultroscan laser using 1,2-diolein (Avanti) as standard.

Data

All results are expressed as means $\pm$ S.E.M. and per 100 mg wet weight of cells, except where noted otherwise, and were analysed by Student's *t* test. Significance was set at $P < 0.05$.

RESULTS

Inositol phospholipid breakdown

The production of free *myo*-inositol and inositol phosphates during incubation of hepatocytes with vasopressin was quantified by gas chromatography. Figure 1(a) shows that, in the absence of Li^+ , vasopressin caused a marked elevation of free *myo*-inositol

Figure 1 Accumulation of free *myo*-inositol in hepatocytes stimulated with vasopressin

Hepatocytes were incubated for 15 min in the absence (a) or presence (b) of 15 mM LiCl before saline/albumin ($\circ$) or 100 nM vasopressin ($\bullet$) was added. At the indicated times, samples were removed, extracted with 15% trichloroacetic acid and derivatized as described in the Experimental section. Results are means $\pm$ S.E.M. for three experiments performed in triplicate. * $P < 0.05$ compared with control.

Figure 2 Accumulation of inositol phosphates in hepatocytes stimulated with vasopressin

Cells were incubated and extracted as described in the legend to Figure 1. Inositol phosphates were converted to free *myo*-inositol by treatment with alkaline phosphatase and derivatized as described in the Experimental section. $\circ$, Control; $\bullet$, vasopressin-stimulated cells. Results are means $\pm$ S.E.M. of three experiments performed in triplicate. $P < 0.05$ compared with control.

of 9.8 nmol/100 mg (210 μM increment above control) at 2 min. (Data refer to the increments above corresponding control values. Intracellular concentrations are calculated on the basis of 47 μl of intracellular water per 100 mg wet weight of cells [18].) The maximal increase was at 15 min (45 nmol/100 mg; 950 μM), and thereafter free *myo*-inositol gradually declined to control values at 60 min. In the presence of 15 mM Li^+ (Figure 1b), free *myo*-inositol remained low, and vasopressin caused no significant changes. These data suggest that there is no agonist stimulation of PtdIns hydrolysis by PLD to form free *myo*-inositol.

To assess the changes in total inositol phosphates, these were dephosphorylated and quantified as free *myo*-inositol. In the absence of Li^+ (Figure 2a), inositol phosphates increased rapidly upon vasopressin stimulation and were significantly elevated at 4 min (increment of 42 nmol/100 mg; 890 μM). The increment at 10 min was 69 nmol/100 mg (1470 μM), and this was largely maintained for the remainder of the incubation.

In the presence of Li^+ (Fig. 2b), much higher basal levels of inositol phosphates were observed, reflecting phosphatase inhibition by Li^+ [19,20]. Vasopressin stimulated a rapid accumulation of inositol phosphates, with a significant increase after 4 min (26 nmol/100 mg; 553 μM). The maximal increment (128 nmol/100 mg; 2720 μM) was reached at 30 min and was maintained for up to 60 min. Re-addition of vasopressin

Figure 3 Accumulation of $^{[3}\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (a), $^{[3}\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ (b) and $^{[3}\text{H}]\text{Ins}(1,3,4)\text{P}_3$ (c) upon addition of saline/albumin (○) or 100 nM vasopressin (●) to hepatocytes pretreated with 15 mM LiCl

Samples were extracted with trichloroacetic acid and subjected to h.p.l.c. analysis. Results are expressed as means $\pm$ S.E.M. of three separate experiments. * $P < 0.05$ compared with control.

(100 nM) at 25 min did not alter the changes in *myo*-inositol or inositol phosphates in the absence or presence of Li^+ (results not shown).

Production of individual inositol phosphates

The preceding studies showed the changes in total inositol phosphates, but not the changes in the individual isomers. Such data are necessary to determine whether or not PtdIns is directly hydrolysed by PLC to $\text{Ins}1\text{P}$. Because the preceding method is not sufficiently sensitive to measure most of these isomers, the hepatocytes were incubated with 0.1 mM *myo*- $^{[3}\text{H}]\text{inositol}$ for 90 min and with 15 mM LiCl for 15 min before addition of saline or hormone, and the labelled inositol phosphate isomers were analysed using h.p.l.c. This preincubation procedure has previously been shown to radiolabel the inositol phospholipids close to isotopic equilibrium [18,21]. Because of the long labelling

Figure 4 Accumulation of $^{[3}\text{H}]\text{Ins}(1,4)\text{P}_2$ (a), $^{[3}\text{H}]\text{Ins}1\text{P}$ (b) and $^{[3}\text{H}]\text{Ins}4\text{P}$ (c) upon addition of saline/albumin (○) or 100 nM vasopressin (●) to cells pretreated with 15 mM LiCl

Samples were extracted with trichloroacetic acid and subjected to h.p.l.c. analysis. Results are expressed as means $\pm$ S.E.M. of three separate experiments. * $P < 0.05$ compared with control.

time, the incubations with hormone or saline were limited to 40 min because cell viability could not be maintained consistently beyond this time.

Figure 3(a) shows that $^{[3}\text{H}]\text{Ins}(1,4,5)\text{P}_3$ significantly increased after 30 s of vasopressin stimulation (the first time point taken). It showed a maximal increase at 1 min, after which it declined to levels that remained 2–4-fold above control. $^{[3}\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ (Figure 3b) peaked upon stimulation with vasopressin at 30 s and declined thereafter to a relatively steady level between 1 and 40 min which was about 2-fold above control. The changes in $^{[3}\text{H}]\text{Ins}(1,3,4)\text{P}_3$ (Figure 3c) paralleled those in $^{[3}\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (Figure 3a) for the first 4 min. However, there was a substantial further increase between 4 and 20 min which was then sustained at about 10-fold above control.

The accumulation of $^{[3}\text{H}]\text{Ins}(1,4)\text{P}_2$ in the presence of vasopressin is illustrated in Figure 4(a). It is apparent that the 5-phosphatase pathway of metabolism of $^{[3}\text{H}]\text{Ins}(1,4,5)\text{P}_3$ was more active than the 3-kinase pathway, since the radioactivity in $^{[3}\text{H}]\text{Ins}(1,4)\text{P}_2$ was in marked excess over that in $^{[3}\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ and $^{[3}\text{H}]\text{Ins}(1,3,4)\text{P}_3$ combined (cf. Figures 3b and 3c). $^{[3}\text{H}]\text{Ins}1\text{P}$

Figure 5 Degradation of [^3H]inositol phospholipids in hepatocytes stimulated with vasopressin

Cells were preincubated with 15 mM LiCl and then incubated with saline/albumin (○) or 100 nM vasopressin (●). Samples were removed at the designated times and analysed for labelled inositol phospholipids as described in the Experimental section. (a) [^3H]PtdIns, (b) [^3H]PtdIns4P and (c) [^3H]PtdIns(4,5) P_2 . Results are means $\pm$ S.E.M. of three experiments. * $P < 0.05$ compared with control.

Ins(1,4) P_2 was significantly elevated after 1 min of vasopressin treatment and showed an almost linear accumulation until 10 min, after which there was little change. The accumulation of [^3H]Ins1P is shown in Figure 4(b). This isomer may be produced by the sequential dephosphorylation of [^3H]Ins(1,3,4) P_3 and Ins P_2 isomers and/or by the direct hydrolysis of PtdIns by PLC. There was no significant change in [^3H]Ins1P during the first 6 min. Thereafter there were small increases above control at 10 and 15 min, and further increases at 20 and 40 min. As will be discussed, these data suggest that there was little or no stimulation of PtdIns hydrolysis by PLC during the first 15 min of vasopressin treatment. Changes in [^3H]Ins4P are shown in Figure 4(c). This was the most prevalent inositol monophosphate isomer and had accumulated to a significant extent after 1 min of vasopressin treatment. The level of this isomer then remained relatively unchanged until 6 min, when a marked accumulation began. When the [^3H]InsPs were summed there was an 8-fold increase

Figure 6 Short-term accumulation (a) and long-term accumulation (b) of PtdOH in hepatocytes incubated with saline/albumin (○) or 100 nM vasopressin (●)

Samples were removed at the indicated times and assayed for PtdOH as described in the Experimental section. Short-term accumulation data are means $\pm$ S.E.M. of three experiments performed in triplicate. Long-term accumulation data are means $\pm$ S.E.M. of five experiments in triplicate. $P < 0.05$ compared with control.

during the first 20 min, but thereafter there was little change (results not shown).

Changes in individual inositol phospholipids

The rapid formation of [^3H]Ins(1,4,5) P_3 observed in the preceding experiments was consistent with the expected rapid hydrolysis of [^3H]PtdIns(4,5) P_2 , but the increase in [^3H]Ins(1,4) P_2 could have been derived from the metabolism of [^3H]Ins(1,4,5) P_3 or the hydrolysis of [^3H]PtdIns4P. To clarify this, the time courses of changes in the different inositol phospholipids were determined using *myo*-[^3H]inositol-labelled cells incubated in the presence of Li $^+$ to limit resynthesis of these phospholipids from [^3H]inositol phosphates. In agreement with Augert et al. [6], Figure 5(c) shows that [^3H]PtdIns(4,5) P_2 decreased almost instantly upon stimulation with vasopressin and then recovered from 30 s onward. From 6 min, the value remained about 25% below control for the rest of the incubation period. Figure 5(b) shows that [^3H]PtdIns4P did not decline so rapidly after vasopressin treatment. A significant decrease was not observed until 2 min, and a maximal decrease occurred at 15 min. The level then remained at about 30% below control. Figure 5(a) illustrates that [^3H]PtdIns also showed a more gradual decrease than [^3H]PtdIns(4,5) P_2 after vasopressin stimulation, which was sustained at about 30% below control from 10 to 40 min of incubation. When the [^3H]inositol phospholipids were summed, there was a 28% decline during the first 15 min, but no further decrease thereafter (results not shown).

Figure 7 Short-term accumulation (a) and long-term accumulation (b) of DAG in hepatocytes incubated with saline/albumin controls (○) or 100 nM vasopressin (●)

Samples were removed at the indicated times and assayed for DAG as described in the Experimental section. Short-term accumulation results are means $\pm$ S.E.M. of three experiments performed in triplicate. Long-term accumulation results are means $\pm$ S.E.M. of five experiments performed in triplicate. * $P < 0.05$ compared with controls.

Production of DAG and PtdOH

To compare the changes in *myo*-inositol and inositol phosphates with those in PtdOH and DAG, we examined production of these lipids over 60 min. After a short delay, there was a rapid increase in PtdOH during the first 2 min (increment above control of 24 nmol/100 mg; 520 μ M) (Figure 6a). Thereafter the PtdOH level remained elevated (average increment of 37 nmol/100 mg; 790 μ M) for up to 60 min (Figure 6b). In contrast, there was a lag of 2 min before DAG was significantly elevated (Figure 7a). Maximal levels of DAG (increment of 30 nmol/100 mg; 640 μ M) were attained by 10 min and were maintained (average increment of 28 nmol/100 mg; 600 μ M) for up to 60 min (Figure 7b). The initial time courses and magnitudes of both PtdOH and DAG production were similar to those observed in earlier studies [4,5].

Effects of ethanol

To determine the role of PLD in the changes in PtdOH and DAG induced in hepatocytes by vasopressin during incubation for 60 min, 75 mM ethanol was added during a 30 min preincubation and again at 15 min to counter a possible loss due to evaporation, and the formation of PtdEtOH and alterations in the other lipids were measured. Figure 8 shows the changes in PtdOH and PtdEtOH. In the absence of ethanol (Figure 8a), PtdOH showed a rapid accumulation on stimulation by vasopressin, consistent with the results of Figure 6. The accumulation was significant by 1 min and maximal by 10 min (43 nmol/100 mg; 660 μ M increment), and was maintained for the entire 60 min incubation. In the presence of ethanol (Figure 8b), the time course of PtdOH

Figure 8 Accumulation of PtdOH in the absence (a) and presence (b) of ethanol, and PtdEtOH accumulation (c)

Cells were preincubated in the absence or presence of 75 mM ethanol for 30 min before additions were made. In the incubations with ethanol, it was re-added at 15 min. ○, Saline/albumin controls; ●, vasopressin-treated cells (100 nM). Results are means $\pm$ S.E.M. of four experiments. * $P < 0.05$ compared with controls.

accumulation was similar, but its magnitude was decreased by 30–40%.

In accord with our earlier report [8], vasopressin caused a rapid accumulation of PtdEtOH in the presence of ethanol, indicative of PLD activation (Figure 8c). The accumulation was significant by 30 s and peaked at 6 min, with no decrease during the rest of the incubation. The steady-state increase above control averaged 4.3 nmol/100 mg, representing an increment of 92 μ M. This was less than the decrease in PtdOH induced by ethanol (average of 11 nmol/100 mg between 10 and 60 min). DAG mass was also determined in the absence and presence of ethanol (Figure 9). DAG accumulation in the absence of ethanol was similar in time course and magnitude to that seen in Figure 7, i.e. there was a lag of 1 min and a slower rise than seen with PtdOH. However, the addition of ethanol was without significant inhibitory effect. Since the re-addition of the alcohol after 15 min of incubation did not alter the levels of PtdEtOH, PtdOH or DAG, it is unlikely that its concentration was limiting.

Figure 9 Accumulation of DAG in the absence (a) and presence (b) of ethanol

Conditions and symbols are the same as those for Figure 8. Results are means $\pm$ S.E.M. of four experiments. * $P < 0.05$ compared with controls.

DISCUSSION

Inositol phospholipid hydrolysis

The present study provided some surprising results. The first was the magnitude of inositol phospholipid breakdown in vasopressin-treated hepatocytes. Our previous chemical measurements of these lipids indicated that, during the first 5 min, the hormone produced a maximal decrease in $\text{PtdIns}(4,5)\text{P}_2$ of approx. 1 nmol/100 mg, but no significant changes in $\text{PtdIns}4\text{P}$ or PtdIns at this time [6]. Although it is well known that $\text{PtdIns}(4,5)\text{P}_2$ is resynthesized from PtdIns via $\text{PtdIns}4\text{P}$ [19] and that $\text{Ins}(1,4,5)\text{P}_3$ is metabolized by several pathways to other inositol phosphates and *myo*-inositol [19,20], the present findings indicate that this resynthesis and metabolism is much greater and more rapid than previously realized. For example, Figures 1 and 2 indicate that the breakdown of inositol phospholipids induced by vasopressin after 15 min resulted in an increase of approx. 110 nmol of inositol phosphates/100 mg in the presence of Li^+ . In comparison, the data of Augert et al. [6] indicate levels in hepatocytes of 1.8, 1.6 and 189 nmol/100 mg for $\text{PtdIns}(4,5)\text{P}_2$, $\text{PtdIns}4\text{P}$ and PtdIns respectively. Although these values appear to be low (cf. 455 nmol/100 mg for PtdIns in whole liver reported by Patton et al. [22]), they illustrate that the inositol phosphates cannot possibly come from $\text{PtdIns}(4,5)\text{P}_2$ and $\text{PtdIns}4\text{P}$, unless these lipids are rapidly resynthesized from PtdIns . Alternatively, PtdIns may be hydrolysed directly, a possibility that is discussed below.

Concerning the extensive further metabolism of $\text{Ins}(1,4,5)\text{P}_3$, this is attested to by the changes in individual [^3H]inositol

phosphates seen in Figures 3 and 4, and by the large increase in *myo*-inositol observed in the absence of Li^+ (Figure 1), which agrees closely with that reported earlier by Prpic et al. [23]. The fact that Li^+ , which inhibits certain inositol phosphate phosphatases [19,20], completely abolished the increase in *myo*-inositol induced by vasopressin, indicates that this increase arose from the inositol phosphates and not from the action of PLD on PtdIns . Although it could be proposed that significant *myo*-inositol accumulation was prevented by a concurrent stimulation of PtdIns synthesis, this seems unlikely since previous measurements of this synthesis indicate that it was increased by vasopressin only slightly (by 30% [18]).

The temporal changes in inositol phospholipids and inositol phosphates in the *myo*-[^3H]inositol-labelled cells are consistent with generally accepted concepts of hormone effects on inositol phospholipid metabolism, i.e. $\text{PtdIns}(4,5)\text{P}_2$ was broken down first, and $\text{Ins}(1,4,5)\text{P}_3$, the primary product, was then metabolized either to $\text{Ins}(1,4)\text{P}_2$ and then $\text{Ins}4\text{P}$ or to $\text{Ins}(1,3,4,5)\text{P}_4$ and then $\text{Ins}(1,3,4)\text{P}_3$ which was further dephosphorylated [19,20]. However, the point of the experiments was to gain information about the possible direct hydrolysis of PtdIns or $\text{PtdIns}4\text{P}$ by PLC. The products of PLC action on PtdIns are $\text{Ins}1\text{P}$ and $\text{Ins}(1:2_{\text{cyc}})\text{P}$ [19]. As shown in Figure 4, vasopressin caused little or no increase in $\text{Ins}1\text{P}$ during the first 15 min, although there were large increases in the other inositol phosphates. These data suggest that there is no direct hydrolysis of PtdIns by PLC during this period. With respect to the accumulation of $\text{Ins}1\text{P}$ between 15 and 40 min, this could have arisen from the metabolism of $\text{Ins}(1,3)\text{P}_2$ and/or $\text{Ins}(1,4)\text{P}_2$ [19,20]. The h.p.l.c. system used to analyse the inositol phosphates did not resolve $\text{Ins}(1,3)\text{P}_2$ from $\text{Ins}(1,4)\text{P}_2$. However, $\text{Ins}(1,3)\text{P}_2$ is a minor metabolite of $\text{Ins}(1,3,4)\text{P}_3$ in liver [20] and its concentration is very much less than that of $\text{Ins}(1,4)\text{P}_2$ in most tissues. In any event, the principal conclusions from the isotopic experiments would not be affected by the presence of $\text{Ins}(1,3)\text{P}_2$ in the $\text{Ins}(1,4)\text{P}_2$ fraction.

With respect to the possible direct hydrolysis of $\text{PtdIns}4\text{P}$ by PLC, Figure 5 shows a 1 min lag in [^3H] $\text{PtdIns}4\text{P}$ breakdown even though there was a very large decrease in [^3H] $\text{Ptd}(4,5)\text{P}_2$ during this time. These data suggest that the PLC that is immediately activated by vasopressin does not act on $\text{PtdIns}4\text{P}$. However, they do not exclude the possibility that another, less-selective, PLC is activated later, perhaps in response to increased cytosolic Ca^{2+} . Because $\text{Ins}(1,4)\text{P}_2$ is also a major product of $\text{Ins}(1,4,5)\text{P}_3$ metabolism, the changes in [^3H] $\text{Ins}(1,4)\text{P}_2$ (Figure 4) cannot indicate whether or not $\text{PtdIns}4\text{P}$ was hydrolysed [there have been no reports of PLD activities towards $\text{PtdIns}4\text{P}$ or $\text{PtdIns}(4,5)\text{P}_2$].

Another finding that emerges from the data of Figures 1–5 is that the hydrolysis of inositol phospholipids decreased after 20 min. For example, there was no significant increase in inositol phosphates beyond 20 min (Figure 2), despite the presence of Li^+ , and the total [^3H]inositol phospholipids and [^3H]inositol phosphates also showed little change during this period (Figures 3–5). Figure 5 also shows that the labelled inositol phospholipids were far from being depleted, in accordance with previous measurements of the unlabelled lipids [6]. Since preincubation of the cells for 90 min did not alter the response to vasopressin, and since re-addition of 100 nM vasopressin at 25 min did not alter the results obtained (not shown), it appears that the activation mechanism for PLC became desensitized. Such desensitization has been noted in other cell types (for references, see [24]) and may involve phosphorylation of signal transduction components by protein kinase C isoenzymes [1]. Interestingly, the chemical data of Augert et al. [6] and the isotopic data of Figure 5 indicate that vasopressin induces a decrease of approx. 25% in cellular

PtdIns at 15 min. If this were largely confined to the plasma membrane, it would represent a major alteration in the lipid composition of this membrane. Desensitization may thus represent a means to preserve the physical integrity of the cell.

Sources of DAG and PtdOH

The extent of inositol phospholipid breakdown, yielding an average increase in inositol phosphates plus *myo*-inositol of 90–110 nmol/100 mg in the absence or presence of Li^+ during 10–40 min, was more than adequate to account for the increase in DAG plus PtdOH (approx. 60 nmol/100 mg) during this period. Presumably the excess DAG and PtdOH was hydrolysed by DAG lipase or utilized for lipid synthesis. We have observed that exogenous DAG is very rapidly metabolized by hepatocytes (S. B. Bocckino, P. F. Blackmore, P. B. Wilson and J. H. Exton, unpublished work), as also reported for T-lymphocytes [25] and NIH 3T3 fibroblasts [26].

As also observed by Bocckino et al. [4,5], there was a delay of 2 min before DAG was increased by vasopressin (Figure 7). This suggests that the DAG formed immediately from PtdIns(4,5) P_2 either is quantitatively insignificant or is rapidly metabolized. The data of Figure 5 indicate that during the first min, only PtdIns(4,5) P_2 is significantly decreased, whereas at 15 min all of the inositol phospholipids are decreased. Since PtdIns(4,5) P_2 shows only a net decrease of 1 nmol/100 mg [6], its hydrolysis would not yield a detectable increase in DAG unless it were concurrently resynthesized from PtdIns4P and PtdIns. Alternatively, PtdIns4P and PtdIns could be directly hydrolysed by PLC.

As discussed above, the hydrolysis of inositol phospholipids in response to vasopressin appears to diminish greatly after 20 min. How, then, are elevated levels of DAG and PtdOH maintained at later incubation times? One explanation is that they are derived from PtdCho and perhaps other phospholipids. This would be consistent with the fatty acid or molecular species composition of the DAG and PtdOH that accumulate in vasopressin-stimulated hepatocytes [4,5,7,27]. Such a conclusion is also supported by the increases in [^3H]alkylacylglycerol and [^3H]alkyl-PtdOH in such cells pre-labelled with [^3H]alkyl-lysoglycerophosphocholine [7]. Although most of the previous analyses of DAG and PtdOH have been performed after only 10 min of incubation with the agonist, we have observed that increased levels of [^3H]alkylacylglycerol and [^3H]alkyl-PtdOH are maintained for at least 60 min (C. J. Allan and J. H. Exton, unpublished work). Another possible reason why the levels of DAG and PtdOH do not decline towards the end of the incubation is that their metabolism/utilization is decreased. Although there is no information on possible changes in DAG lipase activity, the effects of vasopressin on phospholipid synthesis are variable, e.g. stimulatory for PtdIns and phosphatidylethanolamine [18,28] and inhibitory for PtdCho [28,29].

The more rapid increase in PtdOH relative to DAG during the first 2 min of vasopressin stimulation (Figures 6–9) indicates that PtdOH is not formed initially by the action of DAG kinase, in agreement with earlier conclusions [5]. As noted previously [5,8], PtdOH appears to be formed during the first few minutes mainly by the activation of PLD, as revealed by the abrupt rise in PtdEtOH (Figure 8). Since ethanol reduced the level of PtdOH by only 35% between 10 and 60 min (Figure 8) it appears that most PtdOH is not formed by PLD during this time; the likely

alternative source is phosphorylation of DAG. Furthermore, since ethanol did not reduce the level of DAG (Figure 9), its degree of formation from PtdOH is probably small. Although PLD was rapidly activated to form PtdEtOH (Figure 8), the level of this lipid remained constant between 6 and 60 min, despite the re-addition of ethanol at 15 min. Since PtdEtOH is known to accumulate in the liver and other organs of rats treated with ethanol [30], it is considered to be a relatively slowly metabolized compound. If this is the case, the failure of PtdEtOH to increase beyond 6 min suggests that the activation of PLD was short-lived and that, therefore, the contribution of PtdCho, and perhaps other lipids, to the later formation of DAG and PtdOH is mainly via PLC.

In summary, our findings have provided information on the sources of the DAG and PtdOH that are formed during the early phase of vasopressin action. However, the sources of these lipids in the later phase remain unclear. Although there is much evidence that PtdCho is a source of DAG and PtdOH in liver and other tissues [2,3], the magnitude of its breakdown is not known in quantitative terms, and this is presently under investigation.

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