Rapid Changes in Hepatocyte Phosphoinositides Induced by Vasopressin*

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Vasopressin stimulated a 40% decrease in \(^{32}P\)phosphatidylinositol 4,5-bisphosphate and a 15% decrease in \(^{32}P\)phosphatidylinositol within 30 s of addition to hepatocytes prelabeled for 60 min with \(^{32}P\). In hepatocytes prelabeled with \(^{33}P\)inositol for 60 min, vasopressin produced 20% breakdown of phosphatidylinositol and 33% breakdown of phosphatidylinositol 4,5-bisphosphate within 30 s. There was a 40% increase in total phosphatidylinositol 4,5-bisphosphate within 30 s of vasopressin addition. Breakdown of phosphatidylinositol accounted for disappearance of 95% of the inositol lipid label. In hepatocytes from rats labeled in vivo with \(^{3}H\)inositol, vasopressin stimulated 10% loss of labeled phosphatidylinositol.

Loss of \(^{32}P\)phosphatidylinositol due to vasopressin was followed by reincorporation of label to levels greater than control while \(^{32}P\) reuptake into phosphatidylinositol 4,5-bisphosphate did not exceed control values. With \textit{in vitro} \(^{3}H\)inositol-labeled hepatocytes, loss of label from the phosphoinositides was followed by reuptake of tritium label to control levels. In hepatocytes labeled \textit{in vivo} with \(^{3}H\)inositol, reuptake of \(^{3}H\)inositol label did not occur. These data indicate that the hormone-sensitive pool of hepatocyte phosphoinositides can be labeled by both \textit{in vitro} and \textit{in vivo} procedures. Vasopressin induces a rapid decrease of labeled phosphatidylinositol and phosphatidylinositol 4,5-bisphosphate within 30 s.

Phosphatidylinositol breakdown has been linked to the mechanism by which hormones such as the \(\alpha\)-catecholamines and vasopressin elevate cytosolic \(\text{Ca}^{2+}\) (1, 2). The biochemical events underlying hormone-stimulated phosphatidylinositol breakdown have yet to be defined. The hormone-sensitive pool of phosphatidylinositol appears to constitute a small fraction of total cellular phosphatidylinositol (3, 4). Previous studies from this laboratory have shown that the vasopressin-stimulated loss of labeled phosphatidylinositol was localized at the hepatocyte plasma membrane (5). Ligand-stimulated phosphatidylinositol breakdown has also been localized to the plasma membrane in rabbit neutrophils (6). Addition of vasopressin or norepinephrine to isolated liver plasma membranes induced net loss of phosphatidylinositol in the absence of \(\text{Ca}^{2+}\) and in the presence of deoxycholate (7) or cytosol (8). These findings support the hypothesis that vasopressin-stimulated loss of phosphatidylinositol is a direct consequence of receptor activation and not secondary to the elevation of cytosolic \(\text{Ca}^{2+}\) (2).

Recent studies on hepatocytes (9), parotid acinar cells (10), and platelets (11) have shown that \(\text{Ca}^{2+}\)-mobilizing hormones also stimulate a rapid loss of PtdIns-4,5P_2. The majority of these studies have reported changes only in \(^{32}P\) radioactivity which may not accurately reflect the balance of events occurring during hormone stimulation. The present studies have utilized \(^{32}P\), \(^{3}H\)inositol label, as well as measurements of lipid content to define the time course and magnitude of the changes in phosphoinositides induced by vasopressin.

MATERIALS AND METHODS

Hepatocytes were isolated from fed female rats (Charles River Breeding Laboratories, Inc., 160-180 g) by collagenase perfusion as described by Tolbert et al. (12). Hepatocytes were suspended at 8-10 \(\times\) 10^6 cells/ml in phosphate-free Krebs-Ringer bicarbonate buffer and incubated in a Nalgene flask at 37°C in a shaking water bath. 10 \(\mu\)g/ml of \(^{32}P\)P, and 2 \(\mu\)g/ml of \(^{3}H\)inositol were added. The gas phase was 95% \(\text{O}_{2}\), 5% \(\text{CO}_{2}\). At the end of a 60-min labeling period, the hepatocytes were washed twice with 10 ml of fresh buffer and reisolated at 4-5 \(\times\) 10^6 cells/ml. Aliquots of the cell suspension (0.5 ml) were added to plastic tubes with \(\alpha\)-antitrypsin 20 ml/mg of clear supernatant and incubated for the indicated times. Incubations were terminated by the addition of 1.9 ml of methanol/chloroform (2:1) containing 1% concentrated HCl followed by 0.6 ml of chloroform and 0.6 ml of 2 M KCl with vigorous vortexing. Extraction was continued for 1 h at room temperature after which the tubes were centrifuged for 10 min at 5000 rpm (Beckman T-6). The aqueous phase and pellet were removed. The lower chloroform phase was washed once with 1 ml of methanol, 10 mM KH_2PO_4, chloroform (48:47:3, v/v) containing 0.25% HCl. The chloroform phase was evaporated \textit{in vacuo}, resuspended in chloroform, and applied to Silica Gel H plates containing 1% potassium oxalate or Silica Gel 60 precoated thin layer plates (EM reagents). Phospholipids were separated by two-dimensional chromatography employing methanol, chloroform, 4 N NH_4OH, H_2O (90:90:10:19, v/v) in the first dimension (13) and 1-butanol, glacial acetic acid, H_2O (6:1:1, v/v) in the second dimension (14). Labeled phospholipids were visualized by autoradiography following exposure of thin layer plates to Kodak X-Omat film for 12 h. This chromatographic system provides an excellent separation of PtdIns-4,5P_2 from the origin and PtdIns-4P. The \(R_f\) values for PtdIns-4,5P_2 and PtdIns-4P were 0.22 and 0.52, respectively. An unidentified lipid migrates just below PtdIns-4,5P_2. The \(\alpha\) noradrenaline phospholipids including phosphatidylincholine, phosphatidylinositol, phosphatidic acid, phosphatidylserine, and phosphatidylethanolamine migrate above PtdIns-4P and are resolved completely in the second dimension.

Hepatocytes were also isolated from rats injected with 100 \(\mu\)Ci of \(^{3}H\)inositol 18 h prior to isolation of hepatocytes. \(^{3}H\)inositol-labeled phosphoinositides were separated by one-dimensional chromatography utilizing the solvent system methanol, chloroform, 4 N NH_4OH, H_2O (90:90:10:19, v/v). Confirmation of results in the one-dimen-
RESULTS

Time Course of Vasopressin Effect on 32P-labeled Phospholipids—Vasopressin stimulated a rapid loss of 32P label from phosphatidylinositol, PtdIns-4,5P2, and PtdIns-4P in hepatocytes previously labeled in vitro with 32P for 60 min (Fig. 1). At 15 s, 100% of the maximal loss of label from both phosphatidylinositol and PtdIns-4,5P2 was observed (Fig. 1). By 30 s, phosphatidylinositol lost 1700 cpm which represented approximately 10% of the 32P label present in phosphatidylinositol, PtdIns-4,5P2, lost 2030 cpm or 40% of its label, while PtdIns-4P lost 400 cpm or 15% of its label in response to vasopressin stimulation. In agreement with other studies (9, 11), the divalent ionophore, A23187, which elevates cytosolic Ca2+, did not stimulate phosphoinositide breakdown. After 30 s in the presence of 10 mM A23187, the amount of 32P label in phosphatidylinositol, PtdIns-4,5P2, and PtdIns-4P was 99 ± 14, 95 ± 14 and 92 ± 17%, respectively, of the control values ± S.E. for five experiments. These results indicate that elevation of cytosolic Ca2+ is not a sufficient stimulus to initiate rapid loss of 32P label from hepatocyte phosphoinositides.

Continued incubation of hepatocytes with vasopressin resulted in a net increase in [3H]phosphatidylinositol. By 15 min, the amount of label in phosphatidylinositol was 14% greater than in control hepatocytes incubated for the same length of time. Reuptake of 32P label into PtdIns-4,5P2 in the presence of vasopressin occurred over 4 min. Unlike phosphatidylinositol, PtdIns-4,5P2, and PtdIns-4P did not reincorporate 32P label to values greater than control (Fig. 1).

Vasopressin also stimulated an accumulation of labeled phosphatidic acid within 15 s. Maximal formation of labeled phosphatidic acid occurred within 2.5 min followed by a slight decline (Fig. 1). There was no consistent change in the amount of labeled phosphatidylcholine or phosphatidylethanolamine during vasopressin treatment. After 30 s with vasopressin, the amount of label in phosphatidylcholine and phosphatidylethanolamine was 98 ± 6 and 97 ± 6%, respectively, of control values ± S.E. for five experiments. These results indicate that vasopressin stimulated a rapid, transient loss of 32P label from phosphatidylinositol and the polyphosphoinositides while concurrently increasing the formation of labeled phosphatidic acid. Loss of 32P label from phosphatidylinositol in the presence of vasopressin was followed by a stimulated reincorporation of label into phosphatidylinositol to values greater than unstimulated controls. Recovery of PtdIns-4,5P2 in the presence of vasopressin was slower and did not exceed the control levels.

Effect of Vasopressin on in Vitro [3H]Inositol-labeled Hepatocytes—The vasopressin-stimulated resynthesis of phosphatidylinositol utilizing [32P]ATP attenuates the loss of 32P label from phosphatidylinositol after a 1-min exposure to hormone. In an effort to circumvent this, hepatocytes were labeled in vitro by a 60-min incubation with [3H]inositol and thereafter used in experiments with vasopressin. The effects of vasopressin on phosphoinositide breakdown were examined (Fig. 2). The amount of [3H]inositol label incorporated into phosphatidylinositol, PtdIns-4,5P2, and PtdIns-4P was 20,150 ± 1,910, 480 ± 72, and 390 ± 64 cpm, respectively. At least 96% of the incorporated [3H]inositol label was present in phosphatidylinositol. By 30 s, vasopressin stimulated a 21 ± 2% loss of tritium from phosphatidylinositol and a 33 ± 10% loss of label from PtdIns-4,5P2 (Fig. 2). For phosphatidylinositol, this represented a loss of 4,750 cpm as compared to 200 cpm for PtdIns-4,5P2 (Fig. 3). Loss of tritium label from the phosphoinositides was followed by a rapid reincorporation of [3H]inositol label into phosphatidylinositol and PtdIns-4,5P2 to control values. The loss of label from PtdIns-4P was more variable. As in the studies with 32P, the rate and extent of resynthesis was greater for phosphatidylinositol than for PtdIns-4,5P2. However, there was no stimulated uptake of [3H]inositol label into phosphatidylinositol to values greater than control.

Effect of Vasopressin on in Vivo [3H]Inositol-labeled Hepatocytes—Similar rapid effects of vasopressin on phosphatidylinositol breakdown were observed using hepatocytes from rats injected 18 h previously with [3H]inositol. In the experiments shown in Figs. 4 and 5, the amount of [3H]inositol label incorporated into phosphatidylinositol was 6160 cpm. The amount of [3H]inositol label incorporated into PtdIns-4,5P2 was variable. The average incorporation of label for four
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FIG. 2. Time course for vasopressin-stimulated breakdown of in vitro [3H]inositol-labeled phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate. Rat hepatocytes labeled for 60 min in vitro with [3H]inositol and [3H]inositol and [3H]inositol (see Fig. 1) were incubated with or without vasopressin (20 milliunits/ml) for the indicated times. Results are the mean ± S.E. for five separate hepatocyte preparations. Values for vasopressin are based on the percent of control, unstimulated hepatocytes incubated for the same length of time. The counts/min present in the phosphoinositides at the start of the incubation were 20,150 in phosphatidylinositol, 390 in PtdIns-4P, and 480 in PtdIns-4,5P2. *, $p < 0.050$; **, $p < 0.001$.

experiments was approximately 100 cpm precluding measurement of changes in labeled PtdIns-4,5P2. Vasopressin stimulated breakdown of labeled phosphatidylinositol with a maximal effect by 30 s (Fig. 4). In contrast to the in vitro studies, phosphatidylinositol did not regain tritium label in the continued presence of vasopressin. The failure to observe significant resynthesis of labeled phosphatidylinositol with in vivo (Fig. 4) as contrasted to in vitro (Fig. 3) labeled hepatocytes may be a consequence of less precursor [3H]inositol label which amounted to approximately 850 cpm for in vivo as compared to 12,300 cpm for the in vitro situation. This suggests that resynthesis of labeled phosphatidylinositol is determined, in part, by the availability of [3H]inositol.

The low water-soluble radioactivity of in vivo labeled hepatocytes permitted a determination of vasopressin effects on the release of water-soluble label. Vasopressin stimulated a rapid release of water-soluble tritium label that paralleled the initial breakdown of phosphatidylinositol (Fig. 5). Significant effects of vasopressin were evident at 7.5 s, 20% of the total net release of water-soluble [3H]inositol products over 15 min was seen by 7.5 s of hormone addition. This was followed by a continued slow release of label over the next 15-min incubation period. Although the major source of released label was phosphatidylinositol, some label may have been derived from PtdIns-4,5P2.

Effect of Vasopressin on Total Lipid Phosphorus—The ef-
effects of a 30-s stimulation with vasopressin on the total lipid phosphorus content are shown in Table I. There was no significant effect of vasopressin on the amount of PtdIns-4P, phosphatidylethanolamine, or phosphatidylcholine. There was a 10% decrease in the total amount of phosphatidylinositol. However, the amount of PtdIns-4,5P2 increased by 40% during hormone stimulation. Part of the increase in this phospholipid may be accounted for by the decrease in PtdIns-4P and the rest from the decrease in phosphatidylinositol. There was a slight increase in the level of phosphatidic acid.

**Time Course for $^{32}P$ Uptake into Hepatocyte Phosphoinositides and Phosphatic Acid**—The effects of vasopressin on $^{32}P$ incorporation into phosphatidylinositol, PtdIns-4,5P2, and phosphatidic acid are shown in Fig. 6. In these studies, hepatocytes were initially incubated with $^{32}P$ for 5–7 min. Vasopressin produced an immediate inhibition of net $^{32}P$ uptake into PtdIns-4,5P2. At 5 min, however, PtdIns-4,5P2 levels in the presence of vasopressin were similar to control levels. These results indicate that there is a rapid uptake of $^{32}P$ into a pool of PtdIns-4,5P2 sensitive to hormone.

In contrast, uptake of $^{32}P$ label into phosphatidylinositol appeared to be little affected by vasopressin until 2.5 min at which time stimulated uptake into phosphatidylinositol coincident with a decrease in the amount of $^{32}P$ label in phosphatidic acid was observed.

**DISCUSSION**

Interpretation of data on hepatocyte phosphatidylinositol and polyphosphoinositide breakdown utilizing only changes in $^{32}P$ label without measurements of lipid content is complicated by the differential incorporation of isotope into the individual phosphoinositides. Incubation of hepatocytes with $^{32}P$ labeled the hormone-regulated pool of PtdIns-4,5P2 within 5–7 min (Fig. 6). This rapid equilibration of $^{32}P$ label with PtdIns-4,5P2 may be mediated through the sequential phosphorylation of phosphatidylinositol and PtdIns-4P by plasma membrane kinase(s) utilizing $[32P]ATP$ (16–18).

Phosphatidylinositol is thought to be synthesized from $^{32}P$-labeled phosphatidic acid in the endoplasmic reticulum and transferred to intracellular membrane sites, such as the plasma membrane, by the lipid exchange proteins (19, 20). After a 60-min incubation with $^{32}P$, approximately 15% of the incorporated label in phosphatidylinositol was present in a pool which was sensitive to hormone (Fig. 1). Furthermore, net loss of $^{32}P$-labeled phosphatidylinositol was attenuated by the hormone-stimulated uptake of $^{32}P$ into phosphatidylinositol that occurred within 2.5 min of vasopressin stimulation. In contrast, there was no stimulated $^{32}P$ uptake into PtdIns-4,5P2 in the presence of vasopressin (Figs. 1 and 6).

The situation with $[3H]$inositol label differs as much more label is present in phosphatidylinositol than in PtdIns-4,5P2 from hepatocytes labeled in vitro or in vivo with $[3H]$inositol (Figs. 2 and 4). The major effect of vasopressin on $[3H]$inositol-labeled phosphoinositides was to decrease the radioactivity in phosphatidylinositol. The amount of $[3H]$inositol lost from phosphatidylinositol was approximately 20 times greater than from PtdIns-4,5P2 (Fig. 3). The effect of vasopressin on labeled phosphoinositides was rapid with half-maximal loss by 15 s. Clearly, the apparent contribution of a particular phosphoinositide in hormone action depends on the label employed and whether changes in label are accompanied by changes in content.

The present studies confirm the observation of Michell et al. (9) of a rapid vasopressin-induced decrease in the amount of $^{32}P$-labeled PtdIns-4,5P2. Our findings are similar to those recently reported by Thomas et al. (21) who showed that the initial rate of breakdown of $[32P]$phosphatidylinositol was similar to the rate of breakdown of $[32P]$PtdIns-4,5P2 with no apparent lag for the onset of $[32P]$phosphatidylinositol degradation. We did observe with in vitro and in vivo $[3H]$inositol-labeled hepatocytes a secondary sustained loss of lipid label occurring after 5 min of incubation with vasopressin. The rate of loss was approximately 0.4%/min (data not shown) and may indicate that the hepatocyte, like the horse platelet (22), contains multiple pools of phosphatidylinositol. Hormone triggers a rapid initial degradation of phosphatidylinositol followed by a secondary loss of label. The second phase of lipid loss may be a consequence of sustained elevations in cytosolic Ca2+ which block reincorporation of labeled inositol into phosphatidylinositol. Unlike the initial phase of lipid loss which occurs at the plasma membrane (5), the secondary phase is associated with a generalized loss of label from all cellular fractions (23). This secondary effect of vasopressin on phosphatidylinositol was probably measured by Prpic et al. (24) who concluded that phosphatidylinositol breakdown was a late event in hormone action. It appears that these investigators missed the initial event in hormone action. Rhodes et al. (23) and Prpic et al. (24) also concluded that polyphosphoinositide breakdown was secondary to the eleva-
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The present data suggest that loss of labeled phosphatidylinositol is not solely a consequence of its phosphorylation to regenerate PtdIns-4,5P₂ as proposed by Michell et al. (9). The rate of decrease of labeled phosphatidylinositol and PtdIns-4,5P₂, as determined by the time required to attain half-maximal loss of label from the individual phosphoinositides, was similar (Figs. 2 and 3). Furthermore, there was no accumulation of [³²P]inositol in PtdIns-4,5P₂ as would be predicted if labeled phosphatidylinositol were converted to PtdIns-4,5P₂ (Figs. 2 and 4). The specific activity of phosphatidylinositol was 400 cpm/nmol. The specific activity of the hormone-sensitive pool of phosphatidylinositol was approximately 700 cpm/nmol as estimated from the decrease in [³²P]inositol and phosphorus content. These results indicate that the hormone-sensitive pool of phosphatidylinositol has a higher rate of turnover than the total cellular pool of phosphatidylinositol. If one assumes that vasopressin stimulated an initial degradation of PtdIns-4,5P₂ equivalent to the loss of label, then the specific activity of the hormone-sensitive pool of PtdIns-4,5P₂ would be 300 cpm/nmol. Under these conditions, the specific activity of phosphatidylinositol is 2-fold greater than that of PtdIns-4,5P₂, and therefore conversion of phosphatidylinositol to PtdIns-4,5P₂ should result in an increase in the content of labeled PtdIns-4,5P₂. Although PtdIns-4,5P₂ did regain tritium label (Fig. 2), this required at least 5 min and was not in excess of control values. By this time, the amount of labeled phosphatidylinositol had also returned to control values. Finally, in [³²P]uptake studies (Fig. 6), there was no transient increase in [³²P]content in PtdIns-4,5P₂ during vasopressin stimulation as was also expected if the kinase pathway were stimulated to replenish PtdIns-4,5P₂ levels from phosphatidylinositol. In isolated liver membranes (7, 20), vasopressin addition resulted in breakdown of plasma membrane phosphatidylinositol which was a direct effect independent of phosphatidylinositol conversion to PtdIns-4,5P₂. Vasopressin did not increase phosphorylation of phosphatidylinositol to PtdIns-4,5P₂ in the presence of [γ-³²P]ATP under conditions in which breakdown of phosphatidylinositol was seen (27). The data are consistent with a model in which vasopressin produces an apparent activation of a phospholipase C activity which degrades a small pool of plasma membrane phosphatidylinositol and PtdIns-4,5P₂.

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