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Organization of the Receptor-Mediated Phosphoinositide Cycle: Relationship Between Receptor Occupancy and Accession of Phosphatidylinositol

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Abstract We have previously reported the existence of separate hormone-responsive and -unresponsive pools of inositol phospholipids in WRK-1 cells. In order to further explore this concept, we have performed experiments to examine the relationship between the plasma membrane receptor and the pool of phosphatidylinositol (PtdIns) that is metabolized in response to hormonal stimulation. The results support the following conclusions. 1) The amount of PtdIns metabolized in WRK-1 cells in response to vasopressin is proportional to the number of receptors occupied; neither prolonged activation with nor readdition of a submaximal concentration of vasopressin induced the same degree of PtdIns metabolism as a maximal concentration of vasopressin. 2) Dissociation of cytoskeletal structures by incubation with cytochalasin D did not alter the amount of PtdIns accessed during hormonal stimulation. 3) Accession of PtdIns from internal membranes does not depend on internalization and recycling of the receptor; cells incubated in potassium-free medium failed to internalize receptor-ligand complexes, yet they accessed the same amount of PtdIns in response to vasopressin as did control cells. 4) Golgi-mediated phosphatidylinositol transport is not involved in hormone-stimulated phosphoinositide turnover, since brefeldin A, which interferes with Golgi-mediated transport processes, had no effect on the amount of PtdIns accessed during vasopressin stimulation. 5) Phosphoinositide breakdown and compensatory resynthesis is not a closed process; newly synthesized PtdIns is not preferentially localized to a hormone-responsive pool but is generally redistributed between responsive and unresponsive pools. J. Cell. Biochem. 64:382–389. © 1997 Wiley-Liss, Inc.†

Key words: tissue culture; vasopressin; signal transduction; compartmentation; internalization

A number of laboratories have reported the existence of separate hormone-sensitive and -insensitive pools of inositol phospholipids [Monaco and Woods, 1983; Labarca et al., 1985; Schoepp, 1985; Billah and Lapetina, 1982; Rana et al., 1985, 1986; Fain and Berridge, 1979; Vickers and Mustard, 1986; Michell et al., 1988; Monaco and Gershengorn, 1992; Vaziri et al., 1993; Cubitt et al., 1990b]. It has also been well documented that the magnitude of a response to a given agonist is proportional to the degree of receptor occupancy [Holmsen et al., 1984; Kirk et al., 1985; Cory et al., 1987; Ramsdell and Tashjian, 1986; Lynch et al., 1985; Winicov and Gershengorn, 1989; Griendling et al., 1987; Safrany and Nahorski, 1994]. More recently, it has been determined that the absolute amount of total cellular phosphatidylinositol (PtdIns) metabolized in response to activation of the TRH receptor is proportional to the number of receptors occupied [Cubitt et al., 1990a]. Even during prolonged incubations with suboptimal concentrations of agonist, a fraction of the cellular PtdIns could not be metabolized; this fraction, however, was readily accessed during stimulation by maximal concentrations of hormone [Cubitt et al., 1990a]. Identical results were obtained when cells lacking the TRH receptor were transfected with varying concentrations of receptor and subsequently stimulated with agonist [Gershengorn et al., 1994]. Based on these data, it has been hypothesized that the PtdIns metabolized during cycling is located in physical proximity to a given receptor and that,

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following activation of this receptor and metabolism of this PtdIns, the receptor is recycled through the interior of the cell, where it replenishes its supply of PtdIns [Monaco and Gershengorn, 1992].

The relationship between the receptor and compensatory resynthesis of PtdIns has also been explored previously by a number of laboratories [Vaziri et al., 1993; Batty and Downes, 1994; Imai and Gershengorn, 1987a; Sillence and Downes, 1993]. Our laboratory has shown that resynthesis in vasopressin-stimulated WRK-1 cells does not require the continued activation of the receptor [Monaco and Adelson, 1991]. Once PtdIns has been utilized during cycling, its resynthesis is automatic and independent of receptor activation. Since some investigators have observed a PtdIns synthase activity that copurifies with the plasma membrane, it has been suggested that this compensatory resynthesis of PtdIns takes place at the plasma membrane in proximity to the receptor [Vaziri et al., 1993; Galvao and Shayman, 1990; Kinney and Carman, 1990; Imai and Gershengorn, 1987a,b]. However, other investigators, including us, failed to find such an activity in plasma membrane fractions [Williamson and Morre, 1976; Santiago et al., 1993; Morris et al., 1990; Jelsema and Morre, 1978].

In the present study, we continue our studies on hormone-responsive pools of PtdIns. We test the hypothesis that a recycling receptor is required for replenishment of plasma membrane PtdIns by carrying out experiments under conditions which prevent receptor internalization. We also explore the role of the Golgi apparatus and cytoskeleton in facilitating access of the receptor to intracellular PtdIns. In addition, the results of the present study lend further support to the idea that resynthesis does not take place in proximity to the receptor, since the PtdIns resynthesized as a result of cycling is not preferentially localized to a hormone-sensitive pool but rather appears to be distributed to both sensitive and insensitive pools.

MATERIALS AND METHODS

Tissue culture media, trypsin, Dulbecco's phosphate-buffered saline, antibiotic-antimycotic, and glutamine were purchased from GIBCO (Grand Island, NY). Plasticware for tissue culture was from Costar (Cambridge, MA). Anion exchange resin (AG 1-X8, 200–400 mesh, formate form) was from Bio-Rad (Cambridge, MA). [^{3}H]-Ins (15 Ci/mmol) and [^{14}C]-Ins (250 mCi/mmol) were from American Radiolabeled Chemicals (St. Louis, MO). Aquasol-2 was from DuPont–New England Nuclear (Boston, MA). Vasopressin, cytochalasin D, and brefeldin A were purchased from Sigma (St. Louis, MO).

Cell Culture

WRK-1 cells were established from a dimethylbenz(a)anthracene-induced rat mammary tumor as previously described [Kidwell et al., 1978]. The variant used in these experiments (1B) arose spontaneously and has been described previously [Monaco, 1987]. Monolayer cultures were maintained in Ham's F12 medium supplemented with Earle's salts and fetal calf serum (10%), rat serum (2%), penicillin (100 units/ml), Fungizone (0.25 μg/ml), and streptomycin (100 μg/ml). For the experiments described here, the cells were harvested with a solution of 0.05% trypsin, 0.02% EDTA in 0.9% NaCl, and they were replicately plated into 22 mm wells. Details for each experiment appear in the figure and table legends.

Extraction and Analysis of Phospholipids and Ins Phosphates

Lipids and their breakdown products were extracted and analyzed as previously described [Koreh and Monaco, 1986]. The chloroform-extractable radioactivity was quantitated as a single entity. Since greater than 95% of this radioactivity is in the form of PdtIns, it is referred to as such in the text, graphs, and tables.

Depletion of Cellular Potassium

Cells were incubated for 5 min in hypotonic medium (50% minimal essential medium, 50% water), followed by a 1 h incubation in F12 medium containing 100 mM Ins and 10 mM lithium chloride plus or minus potassium chloride. Additional experimental details are described in the figure legends.

RESULTS

Time Course of PtdIns Turnover at Maximal and Submaximal Concentrations of Vasopressin

Cells were prelabeled overnight with ^{3}H-Ins and subsequently incubated for various times with 0.1 M unlabeled Ins and either 10^{-9} M or 10^{-7} M vasopressin. The results are shown in Figure 1. At no time, up to 5 h, did the lower concentration of hormone induce the same de-

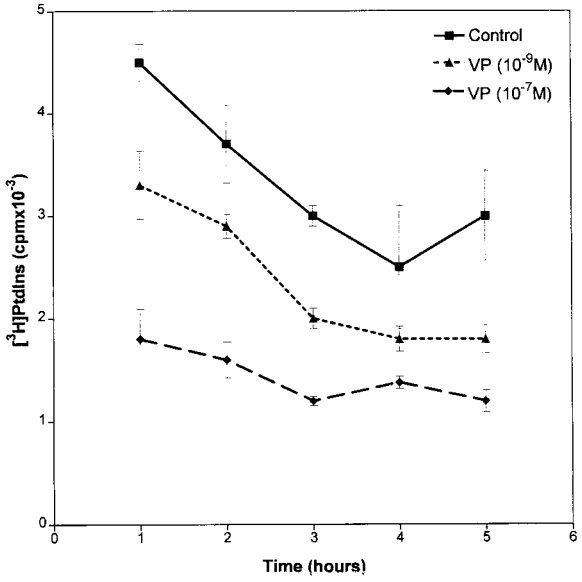

Fig. 1. Turnover of labeled PtdIns in the presence of submaximal and maximal concentrations of vasopressin. Cells were prelabeled for 24 h with [^{3}H]Ins (2.5 μCi/ml) and subsequently incubated in serum-free F12 containing 100 mM Ins and 10 mM lithium chloride plus or minus the concentration of vasopressin (VP) shown for the times indicated. Radioactive PtdIns was quantitated as described in the text. Values shown are the means of six determinations ±1 S.D.

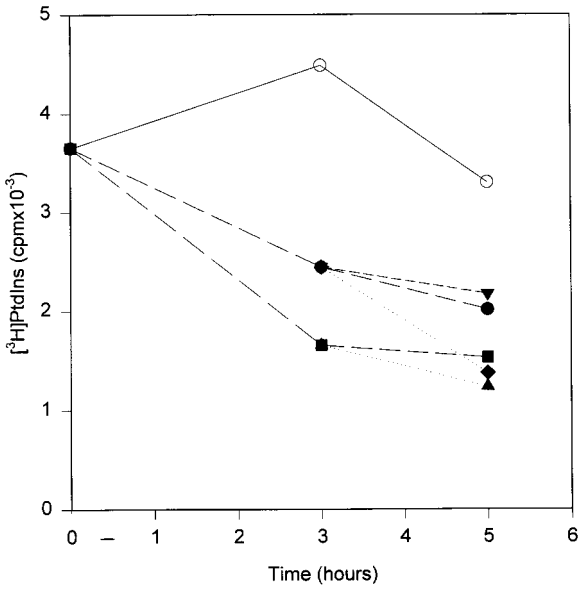

Fig. 2. Effect of readdition of vasopressin on turnover of labeled PtdIns. Experimental methods were as described for Fig. 1, except that at 3 h the cells were washed three times with serum-free F12 followed by readdition of F12 with 100 mM Ins, 10 mM lithium chloride, and the indicated concentration of vasopressin. Symbols are as follows: ○—○, control cells; ●—●, cells incubated with 10^{-9} M vasopressin without a change of medium at 3 hours; ▼—▼, cells incubated with 10^{-9} M vasopressin from 0–3 h, followed by a change of medium and readdition of 10^{-9} M vasopressin from 3–5 h; ◆····◆, cells incubated with 10^{-9} M vasopressin from 0–3 h and 10^{-7} M vasopressin from 3–5 h; ■—■, cells incubated with 10^{-7} M vasopressin without a change of medium at 3 h; ▲····▲, cells incubated with 10^{-7} M vasopressin from 0–3 h, followed by a change of medium and readdition of 10^{-7} M vasopressin from 3–5 h. Results shown are the means of six determinations.

gree of PtdIns turnover as the higher concentration.

Effect of Reapplication of Submaximal Concentrations of Vasopressin

To determine whether or not cells treated for 3 h with a submaximal concentration of vasopressin were capable of further PtdIns turnover, cells were washed after the 3 h of hormone treatment and then incubated for an additional 2 h in the presence of fresh hormone. The results are illustrated in Figure 2. Addition of a low concentration of hormone to cells previously treated with a low concentration had no further effect on PtdIns turnover. However, a maximal concentration of agonist was able to induce further PtdIns turnover, suggesting that the failure of a low concentration of agonist to induce the same degree of PtdIns turnover as a higher concentration was not due to degradation of the hormone or desensitization of the cells.

Effect of Cytochaslasin D on Vasopressin-Induced PtdIns Turnover

[^{3}H]-Ins–prelabeled cells were incubated for 90 min with cytochalasin D prior to stimulation with vasopressin. Figure 3 illustrates the disappearance of radioactivity from cellular PtdIns as a function of time. There was no statistical difference between control cells and those incubated with cytochalasin D with respect to the total amount of PtdIns accessed.

Effect of Potassium Depletion on Vasopressin-Induced PtdIns Turnover

We had previously suggested that replenishment of plasma membrane PtdIns following hormone-induced turnover might result from cycling of the receptor through the interior of the cell [Monaco and Gershengorn, 1992]. Potassium depletion has been shown to prevent downregulation and desensitization of the vasopressin receptor on WRK-1 cells in the presence of agonist [Delafontaine et al., 1987]. Assuming that these were equivalent to the prevention of receptor recycling, we carried out PtdIns turn-

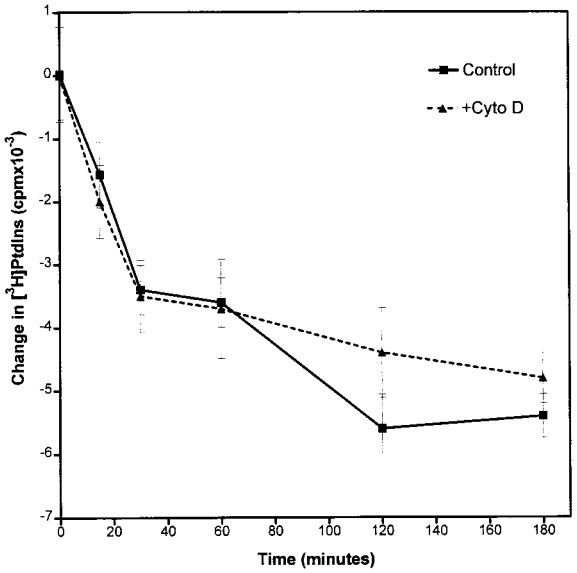

Fig. 3. Effect of cytochalasin D on phosphoinositide cycling. Cells were incubated in growth medium for 24 h with [^{3}H]Ins (2.5 μCi/ml). The medium was then changed to serum-free F12 containing 100 mM Ins and 10 mM lithium chloride plus or minus 1 μM cytochalasin D (Cyto D) for 60 min. Vasopressin (10^{-7} M) was then added to half the samples and the incubation continued for the times indicated. Radioactive PtdIns was quantitated as described in the text. Values shown represent the mean ± S.E.M. of six determinations.

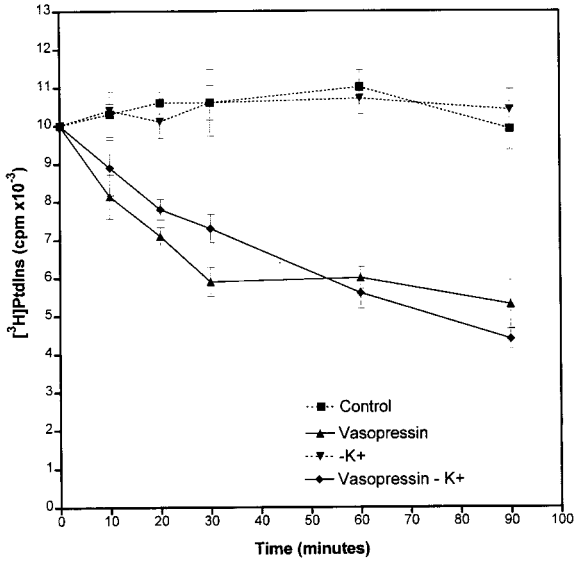

Fig. 4. Effect of potassium depletion of PtdIns turnover. Cells were incubated for 24 h with [^{3}H]Ins (2.5 μCi/ml). Prior to the addition of vasopressin (10^{-7} M), cells were depleted of potassium as described in the text. Cells were then incubated in the presence of 100 mM Ins and 10 mM lithium chloride with or without vasopressin for the times indicated. Radioactive PtdIns was quantitated as described in the text. Values shown are the means ± S.E.M. of six determinations.

over experiments in the absence of cellular potassium. Results are shown in Figure 4. Cells depleted of potassium showed the same response to vasopressin as potassium-replete cells with respect to the total amount of PtdIns accessed during cycling.

Effect of Brefeldin A on Vasopressin-Induced PtdIns Turnover

To determine the role of the Golgi transport apparatus in facilitating hormone-induced PtdIns metabolism, cells were incubated with brefeldin A, an inhibitor of Golgi function. At no concentration of the inhibitor (from 1–10 μg/ml) was lipid turnover attenuated (Fig. 5).

Hormone-Responsiveness of Newly Resynthesized PtdIns

We had previously suggested that the phosphoinositide cycle might be closed, with PtdIns synthesized in response to hormonal activation of cycling being reutilized in the same cycle [Delafontaine et al., 1987]. If this were the case, then it would be expected that all of the newly synthesized PtdIns would be responsive to hormone. To test this hypothesis, cells were prelabeled for 24 h with [^{14}C]-Ins, followed by incubation in the presence or absence of vasopressin with [^{3}H]-Ins for 30 min. The cells were subsequently incubated for an additional 3 h with or without vasopressin in the presence of 100 mM Ins, and the ratio of [^{3}H] to [^{14}C] in the remaining PtdIns was determined. The results, shown in Table I, indicate that the PtdIns newly synthesized in response to vasopressin was present in both hormone-responsive and -unresponsive pools. The increased radioactivity ratio attained initially in the presence of vasopressin was maintained following a second stimulation with vasopressin in the presence of an excess of unlabeled Ins. If newly resynthesized PtdIns were preferentially localized in hormone-sensitive pools, then a decrease in specific activity back to control levels would be expected when prestimulated cells undergo a second round of stimulation in the presence of excess Ins. Similar results were obtained when cells were labeled with [^{3}H]-Ins and [^{32}P]-P_i (data not shown).

DISCUSSION

A number of agonists stimulate the phospholipase C–mediated hydrolysis of $PtdInsP_2$. Following hydrolysis, cellular levels of $PtdInsP_2$ are replenished via conversion of PtdIns to the

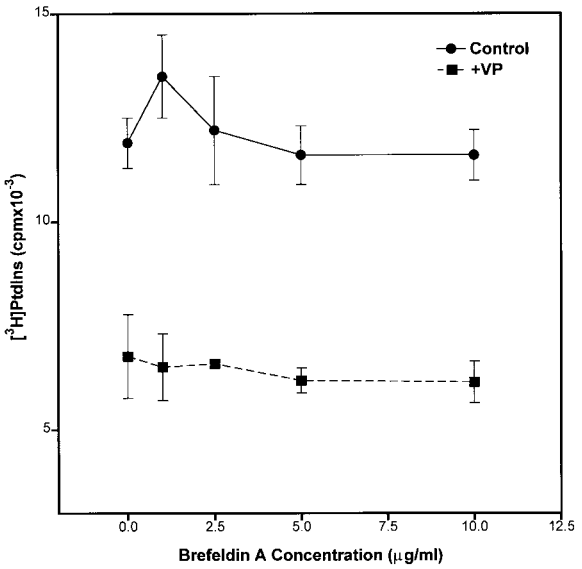

Fig. 5. Effect of brefeldin A on accession of PtdIns during vasopressin-induced cycling. Cells were preincubated for 24 h in growth medium with [^{3}H]Ins (2.5 μCi/ml). The medium was subsequently changed to serum-free F12 containing 100 mM Ins and 10 mM lithium chloride with indicated concentration of brefeldin A plus or minus vasopressin (10^{-7} M). The incubation was continued for 3 h, followed by extraction and quantitation of the radioactive PtdIns as described in the text. Values shown are the means ± 1 S.D. of six determinations.

polyphosphoinositide. As a result, a loss of cellular PtdIns is observed. Using an excess of unlabeled Ins, it is possible to determine the proportion of the total cellular PtdIns involved in this reaction [Delafontaine et al., 1987]. Furthermore, it has been demonstrated by a number of laboratories that some of the cellular PtdIns is resistant to cyclic metabolism in response to agonist [Monaco and Gershengorn, 1992]. Taking this notion a step further, Gershengorn and his colleagues demonstrated that the proportion of cellular PtdIns that participates in cycling is proportional to the number of hormone receptors occupied, regardless of the time of stimulation [Cubitt et al., 1990a; Gershengorn et al., 1994]. In the present study, we confirm this finding (Fig. 1) and extend it to include restimulation of the same cells (Fig. 2). Thus, neither prolonged incubation nor restimulation alter the amount of cellular PtdIns metabolized in response to activation of a fixed number of receptors.

These data suggest an organizational component of cycling which includes the receptor and a specific molecule or molecules of inositol phospholipid. Either the specific receptor occupied determines which PtdIns molecules are metabolized, or the absolute number of receptors occupied dictates the identity of the lipid molecules recruited for cycling. In the first case, it would have to be postulated that an activated receptor was preferentially reactivated upon restimulation. There is some precedence for this increased affinity of an activated receptor under conditions of continual activation [Fishman et al., 1987; Hinkel, 1989]; however, in the case of restimulation, it would have to be postulated that the increase in affinity of the receptor was maintained for the few minutes that the receptors were unoccupied by hormone.

In the second scenario, occupation by agonist of a given number of receptors would result in access to specific PtdIns molecules, perhaps through a mechanism such as tensegrity [Packard, 1986]. Such a mechanism would most likely involve the cytoskeleton. However, experiments carried out in the presence of cytochalasin D, which disrupts cellular microfilaments, demonstrated that there were no differences between the amount of PtdIns accessed in cytochalasin-treated cells and that accessed in control cells (Fig. 3). Previous studies in WRK-1 cells demonstrated a decrease in the accumulation of InsPs in vasopressin-treated cells that had been exposed to cytochalasin D [Ibarrondo et al., 1995]. This was also the case in the experiments reported here (data not shown); however, since there was no difference in the total amount of PtdIns accessed, we presume the difference in InsPs reflects alterations in their metabolism. Similarly, it has been reported that colchicine, which interferes with microtubular structure, has no effect on vasopressin activity in WRK-1 cells [Ibarrondo et al., 1995].

The second question raised regards the mechanism by which PtdIns located on internal cellular membranes is accessed during stimulated cycling. It is not uncommon for cells to metabolize greater than 50% of their PtdIns in response to a calcium-mobilizing hormone. In fact, in WRK-1 cells the combination of vasopressin and bradykinin can cause turnover of 90% of the cellular PtdIns [Monaco et al., 1990]. Since it is clear that not all of this lipid is initially localized at the plasma membrane, there is a question concerning the exact nature of its metabolism. One possibility is that it is transported to the plasma membrane and subsequently converted to $PtdInsP_2$. A second is that PtdIns is converted to $PtdInsP_2$ in the endoplasmic reticulum and then transported to the

TABLE I. Hormone-Responsiveness of PtdIns Synthesized to Replace That Converted to $PtdInsP_2$*

Preincubation addition	Incubation addition	[^{3}H] PtdIns	[^{14}C] PtdIns	$^3H/^{14}C$
None	None	4,252 ± 393	1,777 ± 96	2.38 ± 0.23
None	Vasopressin	3,184 ± 148	1,253 ± 68	2.54 ± 0.19
Vasopressin	None	8,146 ± 690	1,791 ± 151	4.57 ± 0.54
Vasopressin	Vasopressin	5,683 ± 564	1,291 ± 113	4.40 ± 0.38

*Cells were grown for 24 h in medium containing 0.1 μCi/ml [^{14}C]-Ins. [^{3}H]-Ins (10 μCi/ml) was then added for 30 min together with the addition indicated in column 1. Following this incubation, the medium was changed to serum-free F12 containing 100 mM unlabeled Ins and 10 mM lithium chloride plus the addition indicated in column 2. The incubation was continued for an additional 3 h, followed by extraction and quantitation of the radioactive lipids as described in the text. Values shown are the means of sextuplicate determinations plus or minus 1 S.D.

plasma membrane. There is evidence to support this hypothesis [Helms et al., 1991]. A third is that $PtdInsP_2$ at some intracellular location is hydrolyzed and replenished in situ, obviating the need for any transport of PtdIns. There is convincing evidence supporting a nuclear phosphoinositide cycle [Divecha et al., 1993], and $PtdInsP_2$ synthesis has been observed in the endoplasmic reticulum [Helms et al., 1991].

Yet another possibility is that PtdIns is hydrolyzed directly, in situ, as a consequence of agonist stimulation [Wilson et al., 1985; Griendling et al., 1986; Imai and Gershengorn, 1986; Dixon and Hokin, 1985]. However, kinetic data assessing the accumulation and disappearance of the various inositol phosphates argues against this fourth possibility, at least in some model systems [Hughes and Putney, 1989].

We have previously suggested that hormone receptor internalization and cycling through the interior of the cell might provide a mechanism by which intracellular PtdIns is brought to the surface of the cell [Monaco and Gershengorn, 1992]. Previous experiments with WRK-1 cells demonstrated that potassium-free medium prevented a loss of vasopressin receptors from the surface of the cell during stimulation [Delafontaine et al., 1987] and would thus presumably prevent receptors from cycling through the interior of the cell. The experiments reported here in potassium-free medium (Fig. 4) demonstrate that inhibition of receptor internalization has no effect on the amount of PtdIns accessed during hormonal stimulation, negating the hypothesis of a recycling receptor replenishing PtdIns to the plasma membrane. In the previous study in WRK-1 cells, potassium-free medium was reported to attenuate vasopressin-induced InsP accumulation. We noted the same result in our studies; however, this was the result of increased InsP degradation (data not shown) and was not related to a decrease in the amount of PtdIns metabolized.

The subcellular Golgi system provides a means for bringing interior membranes to the surface and as such could replenish plasma membrane PtdIns utilized during cycling. Disruption of the Golgi network, however, had no effect on the amount of cellular PtdIns accessed during vasopressin-induced cycling (Fig. 5).

Lastly, we addressed the question of whether the phosphoinositide cycle was closed or open. In other words, was the PtdIns resynthesized as a result of hormone-induced PtdIns metabolism preferentially redistributed to the same cellular pool of PtdIns? If such were the case, then newly resynthesized PtdIns would remain hormone-sensitive. Our results (Table I) suggest that this is not the case, since the increase in specific activity of PtdIns achieved during an initial stimulation with hormone is maintained during a subsequent stimulation in the presence of excess Ins. Thus, newly synthesized PtdIns is not preferentially relocalized to a hormone-responsive pool but rather generally distributed throughout the cell. These results are in agreement with our previous findings that the phosphoinositide cycle is not closed with respect to Ins, since exogenous Ins and that derived from hormone-induced hydrolysis of Ins lipids exist in the same intracellular pool [Monaco and Moldover, 1995].

In summary, we have provided evidence of a close relationship between receptor occupation and the specific PtdIns metabolized during phosphoinositide cycling. Disruption of the microfilaments of the cytoskeleton does not affect access to these specific pools of lipid. Likewise, neither prevention of receptor recycling nor disruption of the Golgi network interferes with this access. The mechanisms which limit access as a function of the number of receptors occupied as well

as those which confer responsivity to certain PtdIns molecules remain to be determined.

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