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Evidence for a Single Pool of *myo*-Inositol in Hormone-Responsive WRK-1 Cells

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Abstract Previous reports have suggested the existence of at least two pools of cellular *myo*-inositol (Ins); it has been further hypothesized that only one of these pools is utilized during hormone-activated, cyclic phosphatidylinositol (PtdIns) resynthesis. In an effort to investigate this possibility, we have undertaken kinetic studies of Ins metabolism in WRK-1 cells. Our results indicate that a single pool of Ins is involved in both basal and activated PtdIns synthesis. Ins generated by the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) mixes with the existing pool of free Ins and is not used exclusively for resynthesis of PtdIns. © 1995 Wiley-Liss, Inc.*

Key words: *myo*-inositol, cyclic phosphatidylinositol resynthesis, WRK-1 cells, $PtdInsP_2$ hydrolysis

Recent interest in the phosphoinositide signal transduction system has led a number of investigators to examine the biochemistry of *myo*-inositol of (Ins) lipid synthesis and breakdown [Berridge, 1993]. Included in these studies are investigations of the free Ins pool(s) within cells. In several experimental systems, evidence has been presented to support the hypothesis that there are at least two pools of cellular free Ins, one metabolically active and utilized to synthesize Ins lipids, and the second metabolically inert [Greene et al., 1975; Zhu and Eichberg, 1990; Diringer and Rott, 1977; Sigal et al., 1993].

To further examine this possibility, we have carried out a series of experiments utilizing the double label technique first described by MacCallum and his colleagues [1989] to assess Ins specific activities in small numbers of cells under a variety of different conditions. These experiments essentially confirmed the results reported by other laboratories; however, more extensive investigations suggested an alternative interpretation. With the additional data, we have concluded that there is a single, homogeneous pool of free Ins within the cell.

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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 BioRad, Hercules, CA. [^{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. Vasopression was 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 F-12 medium supplemented with Earle's salts and fetal calf serum (10%), rat serum (2%), penicillin (100 U/ml), Fungizone (0.25 μg/ml), and streptomycin (100 μg/ml). For experiments, cells were harvested with a solution of 0.05% trypsin, 0.02% EDTA, in 0.9% NaCl and replicately plated into 22 mm wells.

Details for each experiment appear in the figure and table legends. Unless otherwise noted, cells were grown for several passages in the presence of ^{14}C-Ins in medium containing 100 μM unlabeled Ins (assuming fetal calf serum contains 1 mM Ins) [Berry et al. 1993]. ^{3}H-Ins was added at the start of the experiment, as described in the figure legends. Experiments were performed without any change of medium, except where otherwise noted. We determined that, following 24 h at 37°C in the presence of cells, the serum-containing growth medium was no longer able to stimulate the phosphoinositide cycle, allowing determination of the effects of vasopressin in this medium. Using the values obtained for ^{14}C radioactivity, the relative mass of the Ins-containing compounds was then calculated.

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 phosphatidylinositol (PdtIns), it is referred to as such in the text, figures, and tables. As previously demonstrated, there was no qualitative difference in the behavior of the three phosphoinositide lipids under various experimental conditions [MacCallum et al., 1989; Koreh and Monaco, 1986].

Determination of Specific Activities

Specific activity is defined here as the ratio of ^{3}H to ^{14}C. Crossover of ^{14}C into the tritium channel was 23%; however, this was not considered in the calculations, since there was so little ^{14}C relative to tritium. Crossover of tritium into the ^{14}C channel was 1.3%. This was considered only in calculations where the specific activity ratio was greater than 20.

RESULTS

Time Required for Free Ins and the Ins Lipids to Reach Constant Specific Activity

Cells were grown for several generations in the presence of ^{14}C-Ins before plating in 22 mm plastic wells. The next day, ^{3}H-Ins was added to each well without any change of medium. The cells were harvested after 5, 24, and 48 h, and the radioactivity in free Ins and the phosphoinositides determined. Table I outlines the results. By 48 h, both the free and lipid-associated Ins had achieved a specific activity near or equal to that of the extracellular medium. Effects of vasopressin could be seen only at the early time point (5 h), and had disappeared by 24 h.

Effect of Vasopressin on the Specific Activity of the Free Ins Pool

The ability of vasopressin to lower the specific activity of the free Ins pool at 5 h in the previous experiment (Table I) suggested that there might be more than one pool of free Ins within the cell. In order to examine this further, cells were grown for several generations in the presence of ^{14}C-Ins, preincubated for 2 h with ^{3}H-Ins, and subsequently incubated for varying times with vasopressin. The specific activities of PtdIns and the free Ins pool were then calculated. As shown in Figure 1, treatment with vasopressin caused an increase in Ins lipid specific activity at 30, 60, and 90 min after addition of hormone, whereas the specific activity of the free Ins pool decreased in the presence of hormone. When radioactivity measurements were analyzed separately, we found that the total mass of both PtdIns and Ins (^{14}C-radioactivity) decreased slightly (Fig. 2A and B), whereas more recently transported Ins (^{3}H-radioactivity) decreased to a greater degree in the free Ins pool and increased in the phosphoinositides (Fig. 2C and D). Overall, we observed that vasopressin caused an increase in phosphoinositide-specific activity and a fall in that of free Ins. Considering that the amount of

TABLE I. Time Course of Equilibration of Exogenous Ins With Intracellular Ins in WRK-1 Cells*

		Specific activity	
Time (h)	VP	Ins	PtdIns(s)
5 h	−	22.1 ± 2.6	7.5 ± 0.3
	+	17.6 ± 0.7	8.9 ± 0.4
24 h	−	32.4 ± 1.8	27.0 ± 1.6
	+	31.8 ± 2.0	28.2 ± 2.5
48 h	−	37.4 ± 1.5	37.3 ± 1.5
	+	36.4 ± 2.8	36.5 ± 2.4

*Cells grown for several generations in ^{14}C-Ins were plated in 22 mm wells. Twenty-four hours later, ^{3}H-Ins was added (10 μCi/well) and the specific activities (^{3}H/^{14}C) determined for the phosphoinositides (PtdIns(s)) and Ins at the times indicated. The specific activity of the medium Ins was 43.7 ± 1.0. Values shown are the mean ± 1 SD of 6 determinations.

Ins present in phospholipid is approximately 2.5 times that amount present as free Ins (Fig. 2) [Monaco and Adelson, 1991], it is not surprising that the specific activity of the Ins pool decreased following stimulation, since Ins present as lipid would, upon stimulation, dilute the radioactive Ins newly transported into the cell.

When we compared specific activities of free Ins and stimulated PtdIns, correcting for noncycling PtdIns (30% in this experiment, data not shown), the actual values obtained were very similar to those calculated assuming a single, homogeneous pool of free Ins. Thus the Ins "pool" present in lipid labels much more slowly than free Ins. However, upon stimulation, there is an increase in the labeling of this lipid "pool," while the free pool is diluted with lower specific activity, lipid-derived Ins, thus decreasing the specific activity of free Ins. Similar results were seen in four separate experiments (Table II); the observed changes in specific activities were consistent with a theoretical model invoking a single pool of free Ins which is smaller than that pool present in lipid.

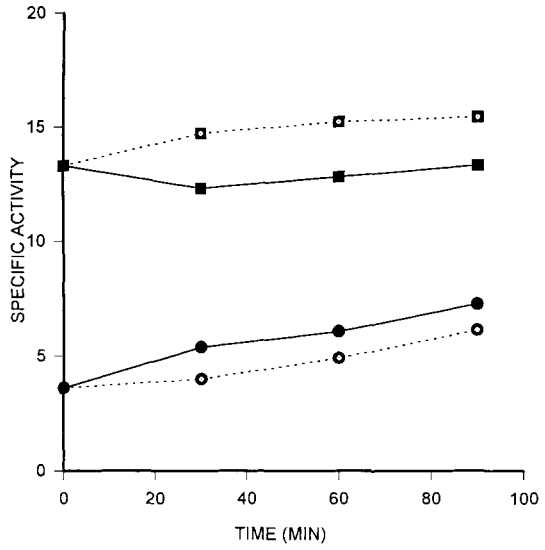

Fig. 1. Specific activities of cellular free Ins (*squares*) and phosphoinositides (*circles*) in control (----, *dotted symbols*) and vasopressin-stimulated (—, *solid symbols*) cells. Cells were grown for 4 to 5 passages in medium containing 0.1 μCi/ml of ^{14}C-Ins. Two hours prior to the start of the experiment, ^{3}H-Ins (20 μCi/ml) was added to each well. The cells were then incubated with or without vasopressin (100 nM) for the times indicated, and the Ins and phosphoinositides quantitated as described in the text. The specific activities shown are the ratios of ^{3}H to ^{14}C radioactivity. Decreases in the specific activity of the free Ins pool are significant, $P = 0.001$ by paired *t*-test; and the increases in the specific activities of the phosphoinositides are significant, $P = 0.005$ (paired *t*-test).

Effect of Lithium on Specific Activities of Ins, Phosphoinositides, and Ins Phosphates

Lithium chloride is known to alter relative levels of Ins-containing compounds in the presence of an agonist. In WRK-1 cells, addition of lithium together with vasopressin causes a shift in radioactive Ins away from lipid and the free pool into Ins phosphates [Monaco and Adelson, 1991]. This is caused by lithium's ability to inhibit Ins phosphatase activity and slow the rate of regeneration of free Ins from Ins phosphates. Thus, in the absence of the lower specific activity Ins generated through phospholipid hydrolysis, and in the presence of stimulated incorporation of the free Ins, the specific activity of the free Ins pool would be expected to increase and approach that of the extracellular pool. Table III compares results from experiments done in the presence and absence of lithium. As predicted, lithium reverses the effects of vasopressin on Ins specific activity. The decrease seen in the absence of lithium is converted to an increase in the presence of lithium.

If there is only a single pool of cellular Ins, then the increase in specific activity observed in the presence of both agonist and lithium would result from the continued uptake of exogenous labeled Ins. Thus, if the continued uptake of Ins were blocked, then the simultaneous addition of agonist and lithium should fail to stimulate a rise in specific activity of free Ins. The experiment illustrated in Table IV demonstrates that this is the case.

Efflux of Radioactive Ins

An alternate approach to differentiating between distinct pools of free Ins involves monitoring the specific activity of Ins which effluxes from the cell. WRK-1 cells were equilibrium labeled with ^{14}C-Ins and incubated for 1 h with ^{3}H-Ins. The cells were then washed with cold PBS and incubated for an additional time in the absence of radioactive Ins with or without vasopressin. Table V illustrates the results of such an experiment. The Ins which leaves the cell has the same specific activity as that which remains in the cell, except when vasopressin is present. In this case, the specific activity of the intracellular Ins drops, as demonstrated previously.

During this experiment, we noted that the Ins leaving the cell appears to do so very rapidly, and probably before the drop in the specific activity of the intracellular Ins. This rapid efflux of 50% of the total Ins did not make sense in

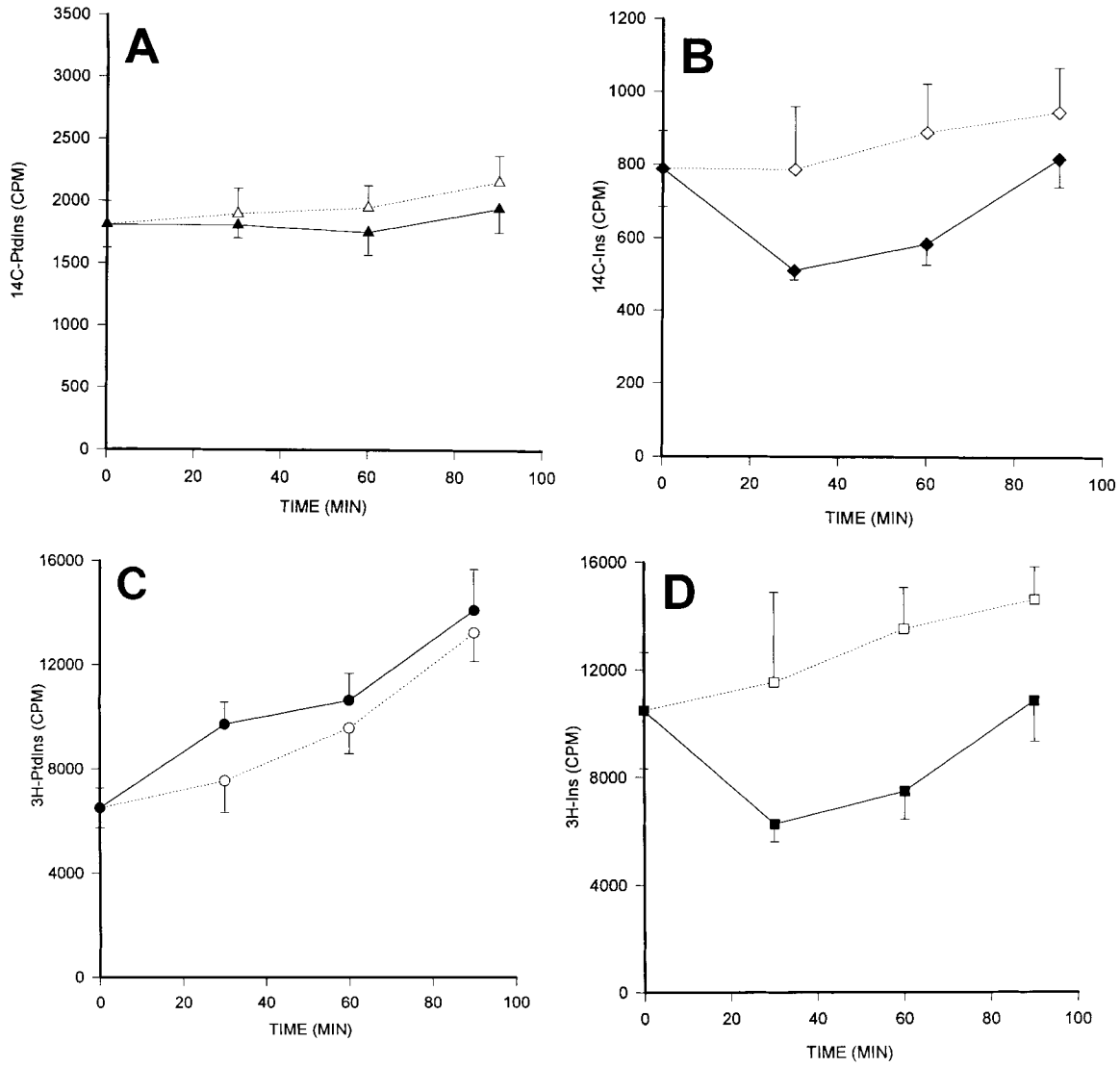

Fig. 2. The effect of vasopressin on short- and long-term labeled free Ins and phosphoinositides (PtdIns). The experiment is that described for Fig. 1; however, values for ^{3}H and ^{14}C radioactivity are plotted separately. **A:** ^{14}C-phosphoinositides. **B:** ^{14}C-Ins. **C:** ^{3}H-phosphoinositides. **D:** ^{3}H-Ins. Controls, dashed lines and open symbols; vasopressin-treated, solid lines and solid symbols. Values shown are the means of six separate determinations ±1 SD.

light of the time it took intracellular Ins to achieve equilibrium with extracellular Ins (see Table I). We therefore tested whether the rapid efflux seen in Table V was an artifact caused by the removal of the medium and washing of the cells before the efflux segment of the experiment. The experiment in Table VI illustrates that this was indeed the case. When influx of radioactive Ins was halted by the addition of a 1,000-fold excess of unlabeled Ins to the medium, there was no subsequent loss of radioactive Ins from the intracellular pool. We also determined that the concentration of extracellular Ins did not affect this efflux (data not shown).

DISCUSSION

A number of previous studies have examined the mechanism of Ins transport into cells [Sigal et al., 1993; Berry et al., 1993; Molitoris et al., 1980; Batty et al., 1993; Yorek et al., 1986; Prpic et al., 1982], with there being general agreement that 1) cells are able to concentrate Ins, and 2) the concentration of Ins within the cell is proportional to the extracellular concentration.

With respect to the intracellular free Ins, it has been suggested by a number of investigators that only a small portion of this is utilized for PtdIns synthesis, and that the remainder is

TABLE II. Comparison of Theoretical and Actual PtdIns Specific Activities*

Experiment No.	PtdIns specific activities	
	Theoretical	Actual
1	6.8	7.2
2	6.6	7.9
3	8.4	7.0
4	10.8	9.1

*Experiments were done as described for Figure 1. In each case, the total PtdIns that cycled was determined by monitoring the level of ^{14}C-PtdIns that remained following treatment with vasopressin in the presence of 100 mM unlabeled Ins. Using these data and assuming a single pool of free Ins, the theoretical specific activity of cellular PtdIns was calculated.

TABLE III. Effect of Lithium Chloride on the Specific Activities of Ins-Containing Compounds*

Addition	Specific activity		
	PtdIns	Ins	InsPs
None	1.99 ± 0.15	15.2 ± 0.90	—[a]
VP	6.07 ± 0.58	11.7 ± 0.73	5.85 ± 0.56
LiCl	1.62 ± 0.07	14.3 ± 0.96	—[a]
VP + LiCl	9.89 ± 0.70	19.8 ± 2.80	6.33 ± 0.51

*Cells were equilibrium-labeled with ^{14}C-Ins. Without a change of medium, ^{3}H-Ins was added for 90 min with the additions noted above. Following this 90 min incubation, Ins containing compounds were quantitated as described in the text. Values shown are the means of 6 determinations ± 1 SD.

[a]Values were too low to calculate a valid specific activity.

metabolically inert. These conclusions are based on a number of different observations:

1. In hepatocytes, that there was a rapid loss of radioactive Ins from prelabeled cells which occurred during the first 20 min of the postincubation period and accounted for approximately 60% of the radiolabeled free Ins [Sigal et al. 1993]. The fall in Ins mass under these same conditions was only 24%. This decrease in Ins specific activity has been interpreted as the efflux of a rapidly equilibrating pool.

2. In sciatic nerve from diabetic rats, the *myo*-Ins concentration was reduced by 30% [Greene et al., 1975], and this was thought to account for the increased accumulation of CDP-DG observed in propranolol-stimulated diabetic nerve [Zhu and Eichberg, 1990]. Addition of Ins to the extracellular medium returned the intracellular Ins levels to normal and reversed the effect on CDP-DG accumulation. Zhu and Eichberg [1990] noted that the concentration of exogenous Ins required to reduce accumulation of CDP-DG (0.3 mM) was lower than that reported to be present endogenously in the diabetic nerve tissue, and they therefore concluded that only a small pool of intracellular Ins was involved in PtdIns synthesis.

TABLE IV. Determination of the Cause of the Increase in Specific Activity of Free Ins in the Presence of Vasopressin and Lithium Chloride*

Addition	Exogenous Ins[a]	Ins specific activity
None	−	8.90 ± 0.14
VP	−	10.84 ± 0.33
None	+	12.10 ± 0.56
VP	+	19.90 ± 4.10

*Cells were equilibrium-labeled with ^{14}C-Ins and incubated for the final 2 h with ^{3}H-Ins, followed by treatment with lithium chloride (10 mM) plus or minus vasopressin, and in the continued presence or absence of the radioactive Ins. The specific activity of free Ins was quantitated as described in the text. Results shown are the means of 2 determinations ± 1 SD.

[a]Radioactive Ins.

3. Studies in chick embryo cells demonstrated that infection with Newcastle-disease virus resulted in a decrease in radioactivity in the free Ins pool, and an increase in PtdIns and its metabolites [Diringer and Rott, 1977]. The authors assumed that the decreased level of radioactive free Ins in the cells resulted from inhibition of uptake. They thus concluded that only a small portion of the intracellular free Ins was involved in PtdIns synthesis, since inhibition of uptake did not affect incorporation into lipid.

WRK-1 cells have an active, hormone-stimulated phosphoinositide cycle [Monaco and Gershengorn, 1992]. We, and others, have often studied this cycle utilizing radioactive Ins substrates. We have previously shown that 1) Ins incorporation into PtdIns in WRK-1 cells is stimulated by vasopressin [Monaco, 1987]; 2) uptake of Ins into cells increases linearly as a function of the extracellular concentration [Monaco and Adelson, 1991]; 3) Ins generated during hormone-induced cycling is reincorporated into PtdIns, except in the presence of high concentrations (10–100 mM) of extracellular unlabeled Ins [Monaco and Adelson, 1991]; and 4) under normal growth conditions (100 μM Ins in the medium), there is more Ins present in the form of lipid than as free Ins [Monaco and Adelson, 1991] and (Fig. 2A and B).

TABLE V. Efflux of Labeled Ins From WRK-1 Cells*

Time (min)	Ins form	VP	^{3}H-Ins (CPM) ($\times 10^{6}$)	^{14}C-Ins (CPM) ($\times 10^{6}$)	Specific activity
0	Free-med.		12.7 ± 3.0	0.055 ± 0.04	230.9
	Free-cell		7254 ± 377	422 ± 14	17.2
	Lipid		1121 ± 32	554 ± 47	2.0
10	Free-med.	−	2,053 ± 241	137 ± 15	15.0
		+	2,058 ± 280	135 ± 9	15.2
	Free-cell	−	3,537 ± 688	228 ± 9	15.5
		+	2,935 ± 477	214 ± 41	13.7
	Lipid	−	1,042 ± 123	391 ± 56	2.7
		+	1,324 ± 207	343 ± 43	3.9
20	Free-med.	−	2,656 ± 357	162 ± 17	16.4
		+	2,310 ± 472	137 ± 28	16.9
	Free-cell	−	3,573 ± 618	219 ± 14	16.3
		+	1,933 ± 398	170 ± 29	11.3
	Lipid	−	1,413 ± 102	445 ± 29	3.2
		+	2,259 ± 329	421 ± 36	5.4

*Cells were equilibrium labeled with ^{14}C-Ins and acutely labeled for 1 h with ^{3}H-Ins. Following the final hour of incubation, cells were washed with PBS and incubated for the times indicated with serum-free F-12 medium containing 10 μM unlabeled Ins, and in the presence or absence of vasopressin (VP). The total radioactivity and specific activity of the Ins present in the medium (free-med.) and in the cells, both free (free-cell) and lipid-associated (lipid), were quantitated as described in the text. Results shown are the means of six separate determinations ± 1 SD.

TABLE VI. Efflux of Labeled Ins From Cells in the Absence of a Change of Medium*

Time (min)	Ins	Change	^{3}H-Ins (CPM)	^{14}C-Ins (CPM)	Specific activity
0	Free	—	8,057 ± 800	527 ± 55	15.3
	Lipid	—	824 ± 85	455 ± 65	1.8
20	Free	Yes	3,984 ± 929	280 ± 49	14.2
	Lipid	Yes	825 ± 159	357 ± 49	2.3
	Free	No	7,999 ± 1,012	484 ± 71	16.5
	Lipid	No	1,059 ± 143	394 ± 31	2.7

*Cells were labeled with radioactive Ins as described for Table IV. Following the labeling period, the further uptake of radioactivity into the cells was prevented by either a change of medium or the addition of 1 mM unlabeled Ins to the medium. Values shown are the means of six separate determinations ± 1 SD.

Previous experiments in a number of different systems have suggested the existence of hormone-sensitive and -insensitive pools of phosphoinositide [Monaco and Gershengorn, 1992]. Such pools have been demonstrated in WRK-1 cells utilizing $^{32}P_i$ [Monaco and Woods, 1983]. We have recently begun a series of experiments with doubly labeled radioactive Ins designed to further examine the concept of such lipid pools. However, multiple pools of free Ins might complicate the interpretation of experimental results, so we undertook the studies described here. The specific activities of both cellular and exogenous free Ins were compared under a variety of experimental conditions. Although much of our data agreed with that reported by other investigators who invoked multiple Ins pools to explain their findings, we were able, with further experimentation, to determine that our results were compatible with a model comprised of only a single homogeneous pool of free Ins. For example, as was reported for hepatocytes [Sigal et al., 1993], there was a rapid fall in cellular free Ins following replacement of the radioactive medium with fresh medium; however, this loss of Ins appeared to be an artifact of cell manipulation (i.e., replacement of the medium), since inhibiting Ins uptake by adding excess unlabeled Ins to the cultures resulted in no loss of radioactive Ins from the cells (Table VI). In addition, the time required for the intracellular Ins pool to equilibrate with exogenous Ins (Table I) argued against rapid equilibration of 50% of the cellular Ins. Likewise, a fall in specific activity of intracellular free Ins following the addition of vasopressin was determined

to result from generation of cellular lipid Ins rather than utilization of a pool of high specific activity Ins for PtdIns synthesis, since lithium was able to attenuate the drop in specific activity by hampering the regeneration of free Ins (Table III). We have previously determined that the pool of Ins generated by metabolism of PtdIns was not utilized exclusively for resynthesis, since exogenous, unlabeled Ins can compete with radioactive Ins for incorporation into PtdIns [Monaco and Adelson, 1991; Downes and Stone, 1986; Cubitt et al., 1990].

With respect to diabetic sciatic nerve [Zhu and Eichberg, 1990], it is not surprising that a relatively low concentration of exogenous Ins (0.3 mM) is able to restore intracellular Ins levels to normal, since cells can concentrate Ins [Molitoris et al., 1980]. In fact, it has been previously demonstrated in this same tissue that 0.2 mM Ins can reverse the decrease in cellular Ins [Greene et al., 1975]. Therefore, there is no need to invoke a second, metabolically active pool of Ins.

The situation described for virally infected chick embryo [Diringer and Rott, 1977] is similar to that observed in hormonally stimulated WRK-1 cells. Free intracellular Ins levels appear to decrease following viral infection of the chick embryo, while levels of radioactive Ins in PtdIns and its metabolites increase. In WRK-1 cells, addition of vasopressin has a similar effect (Fig. 2); in this case, what appears to be inhibition of Ins uptake is in fact an increase in the rate of Ins incorporation into lipid. We have previously shown that, under these conditions, the total amount of radioactive Ins in the cell remains constant, with less in the free Ins pool and more present as lipid and Ins phosphates [Monaco and Adelson, 1991].

Although the data presented here do not rule out the possibility of multiple pools of cellular free Ins, we were unable to generate any convincing data to support such a hypothesis. Whether or not the situation in WRK-1 cells is applicable to other systems remains to be determined.

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