Vol. 101, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1981 Pages 124-130

THYROTROPIN RELEASING HORMONE RAPIDLY ENHANCES [^{32}P]ORTHOPHOSPHATE INCORPORATION INTO PHOSPHATIDIC ACID IN CLONED GH_3 CELLS

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Received June 3,1981

SUMMARY

Thyrotropin releasing hormone (TRH) enhanced incorporation of [^{32}P]orthophosphate into phospholipids in GH_3 cells, cloned rat pituitary cells that produce prolactin. [^{32}P]orthophosphate incorporation into phosphatidic acid was significantly increased 30 sec after TRH addition and attained a steady-state level after 2 min. In contrast, [^{32}P]orthophosphate incorporation into phosphatidylinositol was not significantly increased until 2 min after TRH. [^{32}P]orthophosphate incorporation into phosphatidylcholine and phosphatidylethanolamine were not affected. TRH had no effect on [^{32}P]orthophosphate uptake by GH_3 cells for at least 2 min, but stimulated $^{45}Ca^{2+}$ efflux from GH_3 cells within 15 sec. Since TRH enhancement of [^{32}P]orthophosphate incorporation into phosphatidic acid was temporally associated with its effect on $^{45}Ca^{2+}$ efflux and Ca^{2+} appears to be a critical intracellular regulator of prolactin release, we suggest that acidic phospholipid metabolism may be involved in TRH-induced, Ca^{2+}-mediated prolactin secretion.

INTRODUCTION

A specific and rapid increase in the turnover of the phosphorylinositol moiety of PI[1] has been demonstrated to occur after hormone-receptor interaction in a diverse group of secretory cells (1,2). Since Ca^{2+} has been shown to serve as a critical intracellular regulator of secretion in virtually all of these cell types, it has been suggested that

1. Abbreviations: Thyrotropin releasing hormone, TRH; phosphatidylinositol, PI; phosphatidic acid, PA; phosphatidylcholine, PC; phosphatidylethanolamine, PE; [^{32}P]orthophosphate, [^{32}P]P_i; balanced salt solution, BSS; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Hepes.

0006-291X/81/130124-07$01.00/0

metabolism of PI may be involved in the mechanism by which these hormones regulate Ca^{2+} movements within cells. PA, a precursor of PI but not PI itself, has been shown to have marked Ca^{2+} ionophoretic activity (3,4) and could potentially serve as a Ca^{2+}-mobilizing species. We have recently found that interaction of TRH with its receptor on the plasma membrane of GH_3 cells, cloned rat pituitary cells that produce prolactin, caused mobilization of cell-associated Ca^{2+} which apparently served to couple stimulation by TRH to prolactin secretion (5). It was possible, therefore, that this action of TRH may have involved an effect on phospholipid metabolism.

In this report, we have studied the effect of TRH on $[^{32}P]P_i$ incorporation into phospholipids and have compared it with its effect on $^{45}Ca^{2+}$ efflux from GH_3 cells. We demonstrate a temporal association between enhancement by TRH of $[^{32}P]P_i$ incorporation into PA and of $^{45}Ca^{2+}$ efflux and suggest that acidic phospholipid metabolism may be involved in Ca^{2+} mobilization in GH_3 cells.

MATERIAL AND METHODS

Cell culture. GH_3 cells were grown as monolayer cultures in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum as described (6,7). Prior to an experiment, the cells were harvested with 0.02% EDTA and incubated in BSS - 135 mM NaCl, 4.5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 2 g/l ovalbumin, and 10 mM Hepes, pH 7.4 - for at least 20 min.

Incorporation of $[^{32}P]P_i$ into lipids. Cells were divided into two portions, centrifuged at 180 X g for 5 min, resuspended in fresh BSS containing 0.1 - 0.4 mCi/ml $[^{32}P]P_i$ (carrier-free, New England Nuclear) and placed in a beaker with constant stirring at 26°C. After 30 or 31 min, TRH (final concentration 1 uM, Beckman) was added to one beaker. The incubations were stopped rapidly by pipetting 0.1 ml of cell suspension (1.2-2.0 X 10^6 cells) into 2.4 ml chloroform:methanol (2:1, v/v). The organic phase of the lipid extract was washed with 0.6 ml 2 M KCl - 0.5 M phosphate buffer, pH 7.4, to remove nonlipid phosphorus and exactly 1 ml of the organic phase was taken. The ^{32}P radioactivity of a portion of the organic phase was counted to determin $[^{32}P]P_i$ incorporation into total phospholipids. The remainder was air dried at room temperature and resuspended in a small volume of chloroform: methanol containing 70 ug of the following standards: PA; PI; PC; and PE. This sample was guantitatively transferred onto 0.2 mm thin-layer silica gel 1B plates (J. T. Baker). Phospholipids were separated by one- or two-dimensional chromatography (8). For one-dimensional chromatography, the solvent system was chloroform:methanol:glacial acetic acid:water (25:15:4:2, v/v/v/v). For two-dimensional chromatography the first dimension used the same solvent as the one-dimensional technique and the second dimension used chloroform:methanol:28% ammonia (65:25:5, v/v/v). After development and

drying in air, the plates were placed in a tank saturated with iodine vapor for visualization of the phospholipid areas. After iodine had sublimed from the plates, areas corresponding to the standard phospholipids were cut out, the phospholipids were dissolved in a liquid scintillation cocktail and ^{32}P radioactivity measured. In each experiment,points taken in duplicate varied by less than 10%.

[^{32}P]P_i uptake. Cells were divided into two equal portions, centrifuged at 180 X g for 5 min, resuspended in fresh BSS (0.65 X 10^6 cells/100 ul) and placed in a beaker with constant stirring at 26°C. At zero time, [^{32}P]P_i (40 uCi/ml) with or without TRH (1 uM final concentration) was added to the cell suspension. At the times indicated, the cells were separated by centrifugation at 8,000 X g for 1 min through a layer of silicone in a microfuge (9). ^{32}P radioactivity in the cell pellet was measured in duplicate samples which did not vary by more than 8%.

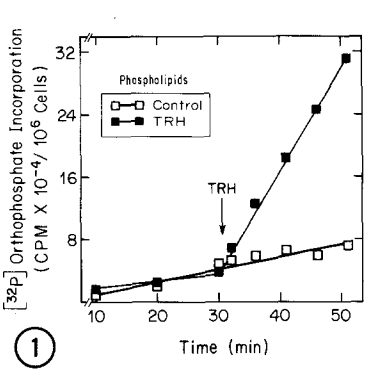
$^{45}Ca^{2+}$ efflux. After the cells were harvested with EDTA, they were resuspended in F-10 medium containing $^{45}Ca^{2+}$ (50 uCi/ml). After 60 min, the cells were washed with BSS, allowed to incubate in BSS for 15 min and resuspended in fresh BSS (1.7 X 10^6 cells/0.1 ml). The cell suspension was incubated at 26°C with constant stirring and TRH (1 uM final concentration) was added at zero time. The cells were separated from the incubation medium as described above and ^{45}Ca radioactivity in the supernatants and in the cell pellets were measured in duplicate. These data are expressed as the fractional efflux (rate constant) which is defined as the fraction of $^{45}Ca^{2+}$ leaving the cells per min (10).

Statistical analysis was performed by *t*-test.

RESULTS AND DISCUSSION

Figure 1 illustrates that there was a constant rate of [^{32}P]P_i incorporation into total phospholipids in control GH_3 cells (1.6 X 10^3 cpm/min/10^6 cells) which persisted for at least 51 min. When TRH was added after 30 min there was an 8-fold increase in the rate of [^{32}P]P_i incorporation (13 X 10^3 cpm/min/10^6 cells) which was already apparent at 2 min, the earliest time measured. This rapid effect on phospholipid metabolism is similar to that described in other cells after secretagogue addition (1,2).

Figure 2 illustrates the effect of TRH, added after 31 min, on [^{32}P]P_i incorporation into PA and PI. There was a very slow, constant rate of [^{32}P]P_i incorporation into PA and PI in control cells. The effect of TRH on [^{32}P]P_i incorporation into PA was very different from its effect on [^{32}P]P_i incorporation into PI. TRH caused an almost immediate increase in incorporation of [^{32}P]P_i into PA. The levels obtained at 5, 10 and 15 sec after TRH addition appeared to demonstrate a progressive increase in [^{32}P]PA; however,



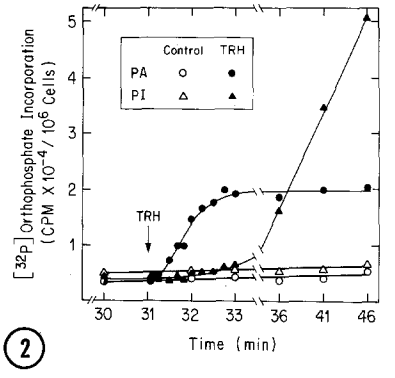


Figure 1 Effect of 1 uM TRH on [32P]Pi incorporation into total phospholipids. [32P]Pi was added at zero time.

Figure 2. Effect of 1 uM TRH on [32P]Pi incorporation into PA and PI.

perhaps since tnese were only duplicate determinations, these values were not significantly different from control. A significant increase in [32P]PA was present after 30 sec and a steady-state level of [32P]PA, 5-fold greater than control, was attained after 2 min and persisted for at least an additional 13 min. [32P]Pi incorporation into PI did not appear to increase until approximately 1 min after TRH addition and was not significantly increased until 2 min after TRH addition. In fact, there may have been a small decrease in [32P]PI up to 50 sec after TRH addition which may represent breakdown of a small amount of prelabeled PI. This would be consistent with the general observation that the enhanced phospholipid metabolism in cells after secretagogue stimulation is initiated by breakdown of PI (1,2). After several min, seen in this experiment beginning with the 6 min point, there was a marked increase in the rate of incorporation of [32P]Pi into PI which persisted for at least an additional 10 min. The maximal rates of [32P]Pi incorporation into PA and PI were virtually identical, however, this rate of incorporation into PA lasted for only 1 min while incorporation into PI persisted for at least 10 min. The similar maximal rates of incorporation into PA and PI suggested that the steps between conversion of PA to PI were not rate-limiting in the metabolism of

Table I

Effect of TRH on $[^{32}P]P_i$ incorporation into individual phospholipids after 5 min.

	$[^{32}P]P_i$ incorporation (CPM X $10^{-4}/10^6$ cells)	
	Control	TRH
Phosphatidic acid	3890±380	25900±1770
Phosphatidylinositol	6160±169	18300±1090
Phosphatidylethanolamine	711±931	513±182
Phosphatidylcholine	610±4	570±4

Cells were incubated in parallel in the presence of $[^{32}P]P_i$. After 30 min, 1 uM TRH was added to one beaker and the incubation was terminated after an additional 5 min (at 35 min).

inositol phospholipids. For comparison, a single previous study involving stimulation of $[^{32}P]P_i$ incorporation into phospholipids in anterior pituitary cells has been reported (11). In this report, a partially purified preparation of "corticotropin releasing factor" was found to increase $[^{32}P]P_i$ incorporation into PA and PI by 40% and 35%, respectively, after 60 min in rat hemipituitary glands.

Table 1 illustrates that the effect of TRH on $[^{32}P]P_i$ incorporation into individual phospholipids was not generalized. After 5 min, TRH increased $[^{32}P]P_i$ incorporation into PA and PI by 6.7-fold and 3-fold, respectively. In contrast, there was no increase in $[^{32}P]P_i$ incorporation into PE or PC in this experiment. In other experiments, there were small increases, up to 2-fold, measurable in $[^{32}P]$PC especially at later time points. This finding that the effect of TRH was mainly limited to acidic phospholipids was also similar to observations in other secretory cells (1,2).

It was possible that the increase in $[^{32}P]P_i$ incorporation into PA might have been due to increased $[^{32}P]P_i$ uptake by the cells. This seemed likely since Martin et al (12) have reported that TRH caused an increase in $[^{32}P]P_i$ uptake by GH_4C_1 cells, a strain of rat pituitary cells related to GH_3 cells. However, in their study, stimulation by TRH of $[^{32}P]P_i$ uptake

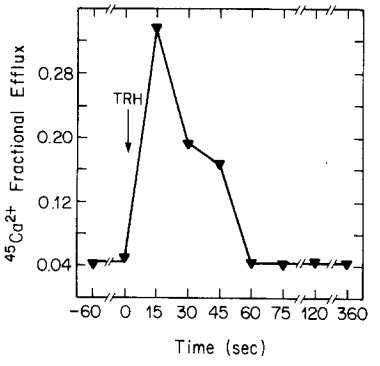


Figure 3 Effect of 1 uM TRH on $^{45}Ca^{2+}$ fractional efflux. Cells were prelabeled with $^{45}Ca^{2+}$ and washed prior to beginning the experimental incubation.

by GH_4C_1 cells was not measurable until 10 min after TRH addition and attained a steady-state level which was only 175% of control after 20 min. We found that TRH had no effect on $[^{32}P]P_i$ uptake by GH_3 cells during a 120 sec incubation (data not shown). Therefore, TRH stimulation of $[^{32}P]P_i$ incorporation into PA was not due to enhanced uptake.

Figure 3 illustrates the time-course of the effect of TRH on the efflux of $^{45}Ca^{2+}$ from GH_3 cells. $^{45}Ca^{2+}$ efflux was studied as a sensitive index of cellular Ca^{2+} metabolism. Basal fractional $^{45}Ca^{2+}$ efflux was 0.044 ± 0.004 min^{-1} (mean±SD). TRH caused a transient increase in the fractional efflux of $^{45}Ca^{2+}$ which was maximal at 15 sec (0.336 min^{-1}), the earliest point measured. Thus, TRH caused a very rapid increase in $^{45}Ca^{2+}$ efflux which was already declining after 15 sec, a time at which a major effect of TRH on acidic phospholipid metabolism was to increase $[^{32}P]P_i$ incorporation into PA.

These data demonstrate that TRH rapidly stimulates phospholipid metabolism in GH_3 cells. The increase in incorporation of $[^{32}P]P_i$ into PA was temporally associated with the effect of TRH on $^{45}Ca^{2+}$ efflux. The data are consistent with the hypothesis (1.2) that enhanced inositol phospholipid metabolism is involved in Ca^{2+} mobilization into or within cells. For GH_3 cells, the close temporal association between stimulation by TRH of $[^{32}P]P_i$ incorporation into

PA, which has a marked Ca^{2+} ionophoretic activity (3,4), and of $^{45}Ca^{2+}$ efflux suggests that PA may mediate Ca^{2+} mobilization. However, since cleavage of the phosphorylinositol moiety from PI most likely preceded the formation of [^{32}P]PA, we cannot exclude the possibility that steps between PI breakdown and PA formation may have been involved in Ca^{2+} mobilization. Since Ca^{2+} appears to be the critical intracellular regulatory (or coupling) factor for prolactin release, we suggest that enhanced PA turnover may be involved in TRH-induced, Ca^{2+}-mediated prolactin secretion.

ACKNOWLEDGMENTS

We wish to thank Ms. Emelda Peck for preparation of this manuscript. This work was supported by Research Grants AM20249 and AA04020 from the National Institutes of Health. M.C.G. is recipient of Research Career Development Award AM00566 from the National Institutes of Health.

REFERENCES

1. Michell, R.H. (1975) Biochem. Biophys. Acta 415: 81-147.
2. Michell, R.H. (1979) Trends in Biochem. Sci. 4: 128-131.
3. Tyson, C.A., Vande Zande, H. and Green, D.E. (1976) J. Biol. Chem. 251: 1326-1332.
4. Serhan, C., Anderson, P., Goodman, E., Dunham, P. and Weissman, G. (1981) J. Biol. Chem. 256: 2736-2741.
5. Gershengorn, M.C., Hoffstein, S.T., Rebecchi, M.J., Geras, E. and Rubin, B.G. (1981) J. Clin. Invest. 67 (in press).
6. Tashjian, A.H., Jr., Yasumura, Y., Levine, L., Sato, G.H. and Parker, M.L. (1968) Endocrinology 82: 342-352.
7. Gershengorn, M.C., Marcus-Samuels, B.E. and Geras, E. (1979) Endocrinology 105: 171-176.
8. Kates, M. (1972) Techniques of Lipidology, pp. 552-553, North Holland/American Elsevier, New York.
9. Finkelstein, M.C. and Adelberg, E.A. (1977) J. Biol. Chem. 252: 7101-7108.
10. Williams, J.A. (1976) J. Physiol. 260: 105-115
11. Hokin, M.R., Hokin, L.E., Saffran, M., Schally, A.V. and Zimmerman, B.U. (1958) J. Biol. Chem. 233: 811-813
12. Martin, T.F.J., Cort, A.M. and Tashjian, A.H., Jr. (1978) J. Biol. Chem. 253: 99-105.