Neurotransmitter-caused increase in [^{3}H]inositol incorporation into phosphatidylinositol de novo synthesis *vs* exchange

Monica Lupu and Yoram Oron*

The Department of Physiology and Pharmacology, The Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv, Israel

Received 6 July 1983

[^{3}H]inositol and $^{32}P_i$ were simultaneously incorporated into rat parotid phosphatidylinositol. The ratio of [^{3}H]/$^{32}P_i$ incorporation dropped dramatically following stimulation with muscarinic or α-adrenergic agonists and returned to control values following the addition of appropriate antagonists. The drop in [^{3}H]/$^{32}P_i$ ratio can be explained by a rapid increase in de- novo synthesis of phosphatidylinositol following its receptor-mediated breakdown. The change in this ratio also provided evidence for the existence of CDP-DG + inositol $\rightleftarrows$ phosphatidylinositol exchange reaction in the intact tissue.

Phosphatidylinositol *αAdrenergic* *Muscarinic* *Receptors* *Rat parotid*

1. INTRODUCTION

Cholinergic and α-adrenergic stimulation of rat parotid slices results in a rapid release of intracellular K^+ [1] and in the increased incorporation of $^{32}P_i$ into phosphatidylinositol (PI) [2,3]. The increased incorporation of radioactive phosphate into PI (phospholipid effect) was first reported in [4] and is the common biochemical event which accompanies stimuli that elicit their response through the mobilization of Ca^{2+} [5,6]. This is preceded, in many instances, by a decrease in tissue PI [7,8].

The disappearance of tissue PI may reflect the breakdown of this phospholipid and this reaction may be involved in the molecular mechanism of the increased permeability of the cell membrane to Ca^{2+} [9]. Inositol can be incorporated into PI by an exchange reaction via the reversal of the biosynthetic pathway [10]. There are, therefore, two possible pathways for the decrease in tissue PI; hydrolytic breakdown catalyzed by a phospholipase or the reversal of the biosynthetic reaction resulting in the accumulation of CDP-diacylglycerol (CDP-DG). Consequently, there are two possible pathways for PI resynthesis; de novo synthesis from diacylglycerol or only the final step-yielding PI from CDP-DG and free inositol.

This work provides evidence for the existence of the exchange reaction in the intact cell, and that the increased incorporation of [^{3}H]inositol into PI, following cholinergic or α-adrenergic stimulation, represents de novo synthesis rather than an increase in the exchange reaction.

2. METHODS

Parotid glands were excised and incubated as in [3,11], except that the bicarbonate was replaced by 25 mM Hepes buffer (pH 7.45) and the gas mixture changed to pure oxygen. Parotid slices were labelled with [^{3}H]inositol (New England Nuclear or Amersham) or $^{32}P_i$ (Nuclear Research Center, Negev) at 100 μCi/ml and 240 μCi/ml, respectively. [^{14}C]Glucose (uniformly labelled) was the product of New England Nuclear.

Following incubation gland slices were homogenized in 1 ml cold water, an aliquot was withdrawn for protein determination according to [12], then trichloroacetic acid was added to a final

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

concentration of 5%. The homogenate was centrifuged (1500 × *g* for 10 min) and the resulting pellet was extracted twice with chloroform:methanol (2:1, v/v). Phospholipids were separated by two-dimensional thin-layer chromatography [chloroform:methanol:acetic acid:H_2O = 25:15:4:2 (by vol.) in the first dimension, 10 cm; chloroform:methanol:NH_4OH:EDTA 5 mM = 30:10:1:1 (by vol.) in the second dimension, 20 cm] on Riedel-De Haen plates (no.37351). The PI spot was identified with iodine vapors by co-chromatography with a marker (Sigma, Israel), scraped out and counted in a liquid scintillation counter (Packard) in a double-label ($^3H/^{32}P$) mode. $^{32}P_i$ uptake was estimated by counting the trichloroacetic acid supernatant of the homogenate. ^{32}P content of the nucleotide fraction was estimated by adsorption on activated charcoal.

All chemicals were of analytical grade. Drugs were obtained from Sigma and Regis. Statistical analyses were performed using paired and unpaired Student's *t*-test.

3. RESULTS AND DISCUSSION

Rat parotid slices incorporated [^{3}H]inositol into PI. The rate of incorporation was constant up to 180 min incubation. When gland slices were exposed to carbamylcholine and then to atropine and [^{3}H]inositol, the rate of label incorporation into PI was significantly higher than that of control gland slices (fig.1). Similar results were obtained following α-adrenergic stimulation and blockade (not shown). This increase was compatible with cholinergic breakdown of PI [13] and its subsequent re-synthesis. Cholinergic stimulation (carbamylcholine 0.1 mM, 20 min) resulted in a marked increase in the incorporation of both [^{3}H]inositol (280 ± 110% over controls, 7 expt) and $^{32}P_i$ (550 ± 60% over controls, 7 expt) into tissue PI. The increased incorporation of [^{3}H]inositol into PI was investigated using a double-label technique. Parotid slices were incubated with [^{3}H]inositol and $^{32}P_i$ simultaneously and the incorporation into PI assayed in control glands and following α-adrenergic or cholinergic stimulation. The relative incorporation of inositol and phosphate into PI was defined by the ratio (*R*) of ^{3}H and ^{32}P counts incorporated into the same chromatographic spot of PI. This ratio decreased dramatically upon the

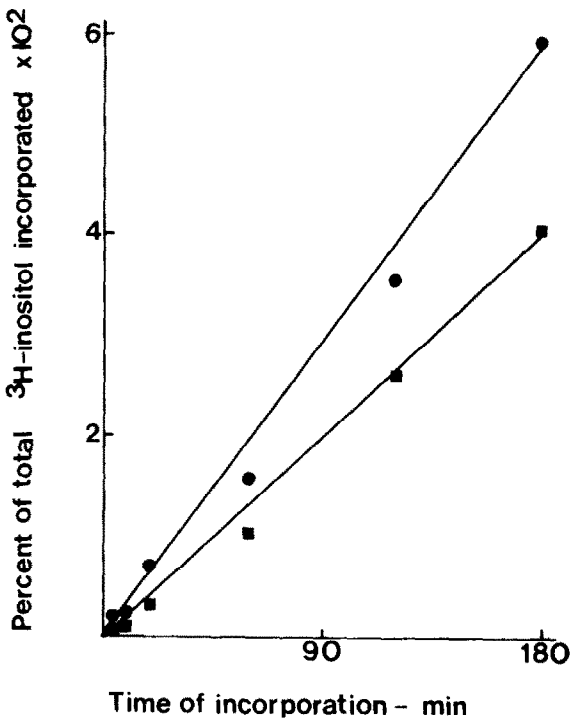

Fig.1. The incorporation of [^{3}H]inositol into rat parotid slices. Parotid slices were prepared and incubated as in section 2. The tissue was incubated either without any additions (squares) or with carbamylcholine (100 μM, circles) for 30 min. At that time atropine (10 μM) was added with [^{3}H]inositol (100 μCi/ml) and its incorporation into PI was determined at the indicated times. The results are the mean of 3 expt and are presented as % total label incorporated into PI. Paired Student's *t*-test was used and the incorporation was significantly higher ($p < 0.05$) in tissue pre-exposed to carbamylcholine.

exposure of gland slices to carbamylcholine, or epinephrine and propranolol (to ensure α-adrenergic stimulation). The decrease in *R* was blocked by the specific muscarinic and α-adrenergic blockers, atropine and phentolamine, respectively. The antagonists alone did not affect the ratio (fig.2). The decrease in *R* caused by carbamylcholine could be reversed by the addition of atropine (fig.3). The initial rise in *R* during the first 45 min of incubation with the radio-labels could be explained by a more rapid equilibration of the intracellular phosphate pool with the external label.

The decrease in the ratio of incorporation of [^{3}H]inositol/$^{32}P_i$ into PI could be interpreted in

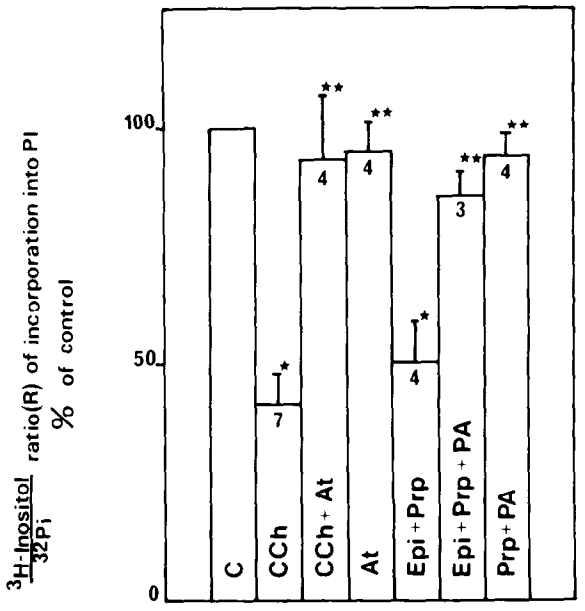

Fig.2. Decrease in the ratio of [^{3}H]inositol/^{32}P$_i$ (R) incorporation into PI in rat parotid slices upon α-adrenergic or cholinergic stimulation. Parotid slices were incubated in the presence of [^{3}H]inositol (100 μCi/ml) and ^{32}P$_i$ (250 μCi/ml) for 20 min. Tissue samples were processed and analyzed for the incorporation of both isotopes into PI as in section 2. The ratio (R) of incorporation in control tissue (no additions) was assigned the value of 100% and the other group treatments were normalized accordingly. The bars represent the means ± SEM of 3–7 expt performed in triplicate. Unpaired Student's t-test was used to calculate the significance of differences between the experimental systems and the control; * $p < 0.01$; ** not significantly different from control.

several ways. An increase in the specific radioactivity of the cellular ATP pool due to the cholinergic or α-adrenergic K^+ release and the stimulation of Na^+/K^+-ATPase, or an increase in $^{32}P_i$ uptake could provide a trivial explanation. However, no increase in the specific radioactivity of ATP upon α-adrenergic stimulation was reported in [2]. Moreover, we measured $^{32}P_i$ uptake and found that it decreased, rather than increased upon cholinergic stimulation (not shown). We have also conducted experiments similar to those shown in fig.2 in the absence of Ca^{2+} and in the presence of EGTA in the medium (to abolish K^+ efflux and the associated activation of Na^+/K^+-ATPase), as well as following the removal of extracellular label, which prevented any change in the specific radioactivities of the intracellular pools. In both types of experiments the results were qualitatively unchanged (table 1). The decrease in R could also be the result of a large decrease in the specific radioactivity of the in-

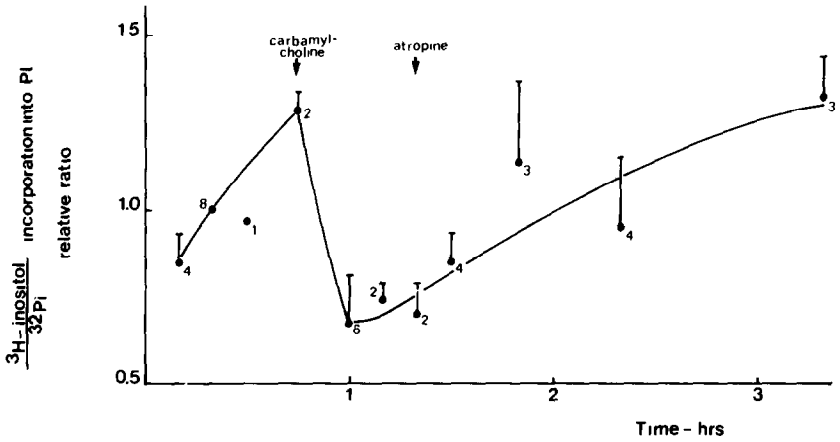

Fig.3. The kinetics of the ratio of incorporation of [^{3}H]inositol/^{32}P$_i$ in rat parotid gland. Gland slices were incubated with [^{3}H]inositol and ^{32}P$_i$. Carbamylcholine (100 μM) was added at 45 min incubation, and atropine (10 μM) at 80 min. At the indicated times samples were withdrawn and the ratio of [^{3}H]inositol/^{32}P$_i$ incorporation into PI was determined as in fig.2 and section 2. All results were normalized to the value of R at 20 min incorporation, which has been assigned an arbitrary value of 100%. The points represent the means ± SEM of a number of experiments given at each point.

Table 1

The decrease in the ratio of incorporation of [^{3}H]inositol/$^{32}P_i$ into PI in rat parotid slices in the absence of Ca^{2+}, or after the removal of the radioactive label

Treatment (*N*)	*R* = ratio of incorporation of $^3H/^{32}P$ into PI		
	Control	Carbamyl-choline	*p*
None (6)	12.0 ± 1.9	5.2 ± 0.6	<0.01
No Ca^{2+} + EGTA (6)	22.7 ± 1.6	5.9 ± 0.5	<0.01
Wash (6)	10.0 ± 0.4	3.4 ± 0.2	<0.01

Parotid slices were incubated as described in fig.2 (no treatment), without Ca^{2+} and in the presence of 1 mM EGTA, or were washed thoroughly with non-radioactive medium before the addition of drugs. Carbamylcholine was 100 μM. The results are the means ± SEM of 3 expt. Statistical significance was determined by unpaired Student's *t*-test

tracellular pool of inositol. This could be effected by a massive synthesis of inositol. [^{14}C]Glucose was incorporated only slightly into PI (most probably into the glycerol moiety). This incorporation did not increase upon cholinergic stimulation.

It appears, therefore, that the only valid interpretation of changes in *R* is a change in the relative rates of de novo synthesis and exchange reactions. Two major conclusions can be drawn from these results:

(1) The CDP-DG + inositol $\rightleftharpoons$ PI + CMP exchange reaction was previously demonstrated in cell-free preparations [10,14], and by inference, in the intact cell [15]. To the best of our knowledge, this is the first direct demonstration of this reaction in the intact cell.

(2) The disappearance of PI upon hormonal stimulation is a common early event in many tissues. Our results can rule out the reversal of the biosynthetic pathway as a possible mechanism. The breakdown of PI by a phospholipase C-type enzyme, the concomitant release of diacylglycerol, and the subsequent de novo synthesis of PI appear to be more likely. The reports of the accumulation of inositol–phosphate [16], particularly when phosphatase is inhibited by Li ([17–19], and Hokin-Neaverson and Eisenberg, personal communication) are thus confirmed. The overall relevance of the PI breakdown to the calcium-gating process remains, however, to be elucidated.

ACKNOWLEDGEMENT

This project was supported in part by a Schreiber Foundation grant.

REFERENCES

[1] Batzri, S., Selinger, Z., Schramm, M. and Robinovich, M.R. (1973) J. Biol. Chem. 248, 361–368.
[2] Oron, Y., Lowe, M. and Selinger, Z. (1973) FEBS Lett. 34, 198–200.
[3] Oron, Y., Lowe, M. and Selinger, Z. (1975) Mol. Pharmacol. 11, 79–86.
[4] Hokin, M.R. and Hokin, L.E. (1953) J. Biol. Chem. 203, 967–977.
[5] Michell, R.M. (1975) Biochim. Biophys. Acta 415, 81–147.
[6] Berridge, M.J. (1981) Mol. Cell. Endocrinol. 24, 115–140.
[7] Hokin-Neaverson, M.R. (1974) Biochem. Biophys. Res. Commun. 58, 763–768.
[8] Jones, L.M., Cockroft, S. and Michell, R.H. (1979) Biochem. J. 182, 669–676.
[9] Michell, R.H. (1979) Trends Biochem. Sci. 4, 128–131.
[10] Hokin-Neaverson, M.R., Saghedian, D.W., Harris, D.W. and Merrin, J.S. (1978) in: Cyclitols and Phosphoinositides (Wells, W.W. and Eisenberg, F. jr eds) pp.349–360, Academic Press, New York.
[11] Oron, Y., Kellogg, J. and Larner, J. (1978) Mol. Pharmacol. 14, 1018–1030.
[12] Lowry, O.H., Rosebrough, N.J., Farr, J.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[13] Jones, L.M. and Michell, R.H. (1974) Biochem. J. 142, 583–590.
[14] Jelsema, C.L. and More, D.L. (1978) J. Biol. Chem. 253, 7960–7971.
[15] Tolbert, M.E.M., White, A.C., Aspry, K., Cutts, J. and Fain, J.N. (1980) J. Biol. Chem. 255, 1938–1944.
[16] Fain, J.N. and Berridge, M.J. (1979) Biochem. J. 178, 45–58.
[17] Hallcher, L.M. and Sherman, W.R. (1980) J. Biol. Chem. 255, 10896–10901.
[18] Honchar, M.P., Olney, J.W. and Sherman, W.R. (1983) Science 220, 323–325.
[19] Berridge, M.J., Downes, P.C. and Hanley, M.R. (1982) Biochem. J. 206, 587–595.