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INVOLVEMENT OF THE PHOSPHOINOSITOL (PI) SYSTEM IN THE MECHANISM OF HORMONAL IMPRINTING

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Certain components of the phosphoinositol (PI) system are present in the unicellular Tetrahymena. Treatment of Tetrahymena with insulin did not alter the relative proportions of the examined six phospholipid components (PIP_2, PIP, phosphatidylcholine,phosphatidylethanolamine, PI, PA), but the primary interaction (imprinting) with insulin accounted for an about 75% decrease in the PIP_2 - level and an about 20% incrase in the phosphatidylethanolamine level. The experimental results strongly suggest that hormonal imprinting accounted for adjustment of the second messenger systems of Tetrahymena to an energy saving level.

In higher organisms the primary interaction of a signal (hormone) molecule with the target cell induces , in the genetically encoded, yet prenatally still plastic cellular binding structure (receptor), a hormonal imprinting, which accounts for maturation of the receptor and for establishment of the cellular response characteristic of adulthood (1,2). The same mechanism of hormonal imprinting can also take place in unicellular organisms(3), except that instead of the genetically encoded hormone binding site a non-specific membrane structure, which is able to bind the hormone and to alter, as a result, the binding capacity and response of the cell, is amplified by the hormonal influence. Since unicellulars are short-lived, the change induced by hormonal imprinting can be assessed only in the daughter cells, in which the "memory" of primary interaction may persist over as many as 600 generations (4). Depending on the nature of the hormone, the mechanism of imprinting involves different second messenger systems, which are also present in Protozoa, such as the cyclic AMP-adenylate cyclase, cyclase GMP-guanylate cyclase and Ca-calmodulin systems (5-10). Inositol-phospholipids, too, act as second messengers in the mediation of extracellular signals to the cytoplasmic structures and they also collaborate in processes regulated by other second messengers.

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It is known that eukaryotic unicellulars possess a phosphoinositol (PI) system. The green alga Dunaliella salina averts the adverse effects of environmental osmotic changes by processes controlled by rapid PI degradation (11). Inositol-glycerolipids cover about 10% of the total phospholipid content of the unicellular Paramecium (12). It has been suggested that the transmembraneous signals acting on Paramecium stimulate inositol-lipid-specific phospholipase A2 activity, which gives rise to release of arachidic acid and in turn to an increase in prostaglandin synthesis (13). Prostaglandin elevation precipitates a cascade-like series of reactions,which elicit several physiological responses. Earlier observations on inhibition of hormonal imprinting in Tetrahymena by neomycin (14),a known inhibitor of PI turnover (15), indicated involvement of the PI system in hormonal imprinting. With these facts in mind, we examined whether the extent of inositol-phospholipid degradation differed between Tetrahymena populations imprinted and not imprinted with hormone (insulin).

MATERIALS AND METHODS

Tetrahymena pyriformis GL cells, cultured in 0.1 per cent yeast extract containing 1.0 per cent Bacto Tryptone medium (Difco,Michigan, U:S.A.) at 28^{o}C, were used in the logarithmic phase of growth.

Part of the mass cultures were not treated, to serve as control, part were pretreated (imprinted) with 10^{-6}M insulin (Insulin Semilente MC, Novo, Copenhagen, Denmark) for 1 h, after which they were washed in plain medium and returned to plain medium for 24 h. Subsequently 150 ml samples of culture suspension (10^{5} cells/ml) were treated with 0.1 ml ^{32}P (sodium orthophosphate) solution of 400 MBq/ml activity (Izinta,Budapest) for 90 min at 28^{o}C, were washed in four changes of plain medium, and were assigned to two groups, of which one was not treated (control group), whereas the other was treated with 10^{-6}M insulin.

The treated and not treated cultures were equally sampled at the beginning of insulin exposure (0 time), and at 30 sec, 60 sec, 10 min and 30 min after it. Five ml sample was combined with 5 ml 10% aqueous solution of 4^{o}C trichloracetic acid, and was incubated for 20 min at 4^{o}C. After incubation the samples were centrifuged and the sediment was extracted with 2 ml acid chloroform-methanol solution (chloroform: methanol:HCl = 10:20:1) at room temperature for 15 min. The extract was partitioned by addition of 0.66 ml chloroform and 0.66 ml water (12), and the aqueous phase was dried in N-flow.

The phospholipid components of the samples were separated by one-dimensional two-step thin-layer chromatography,as proposed by Medh and Weigel and the chromatogram was covered with an X-ray film (Medifort RP, FORTE, Vác, Hungary), which was developed after exposure for 36 h, to detect the phospholipid spots. These were, after identification,scraped into scintillation cuvettes and were, after addition of Turner's scintillation cocktail, assayed for radioactivity in a Beckmann LS 9000 scintillation counter.

PIP2 (L-O-phosphatidylininositol-4,5 diphoshpate, triammunium salt) and PIP (L-O-phosphatidylinositol-4-monophasphate, diammoni-

um salt) (Calbiochem, Luzern, Switzerland) were used as reference preparations for identification of the chromatographically separated phospholipids.

In every case, three replica assays were performed, and the values shown in the Figures represent means of these. The quantity of each detected lipid was expressed in per cents related to total ^{32}P-incorporation. Six lipids were detected and analysed for quantitative relations, such as 1) phosphatidic acid (PA): 2)phosphatidylinositol (PI): 3) PIP: 4) PIP_2 5) phospatidylcholine (PC) and 6) phospatidylethanolamine (PE).

RESULTS

Control cultures: At 0 time, PE was present at the highest concentration (48% of total activity), and PIP_2 (19.5%), PI, PC (13.8% each), PIP (3.8) and PA (2.8%) followed in the falling sequence. Insulin treatment accounted for an appreciable decrease in the PIP_2 level at 30 sec, and for a significant increase in the levels of PE and PIP_2 at 30 min (Fig.1, 2).

Insulin-imprinted cultures: At 0-time (i.e.24 h after imprinting) the level of PIP_2 decreased considerably (from 19.5 to 4.8%) relative to the control, whereas the PE-level increased considerably over the control (from 48 to 60.5%). Reexposure of the imprinted

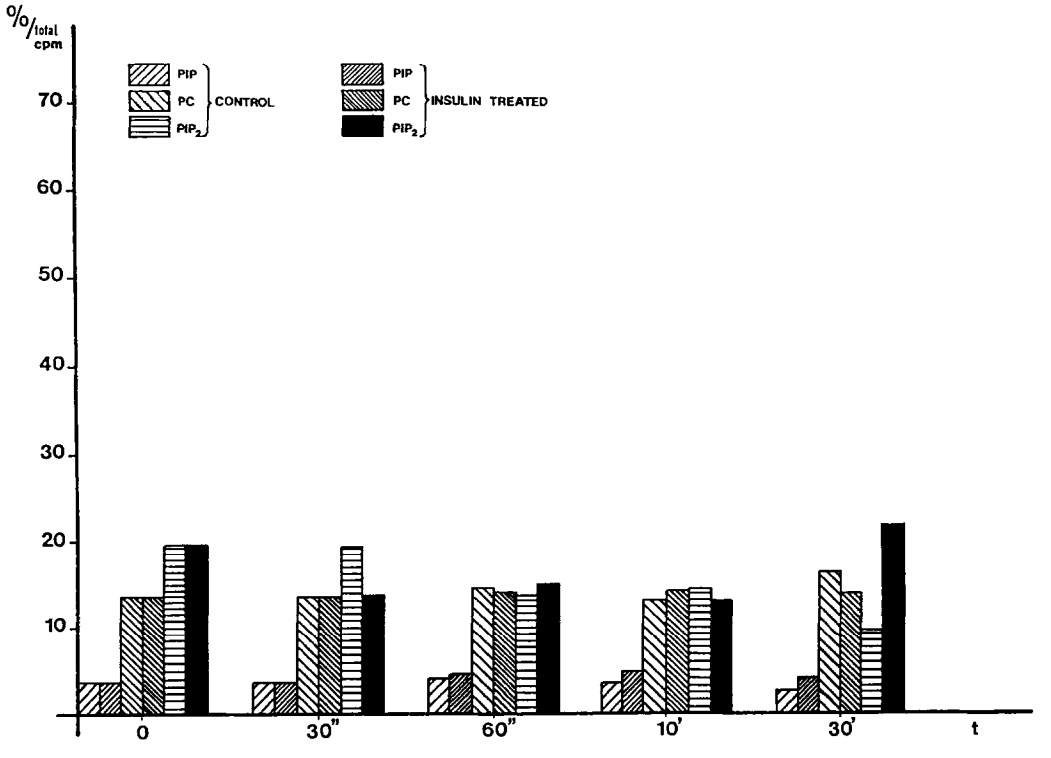

Fig. 1. PIP_2 , PIP and phosphatidylcholine levels in control (not pretreated, not treated) and insulin treated (not pretreated) Tetrahymena.

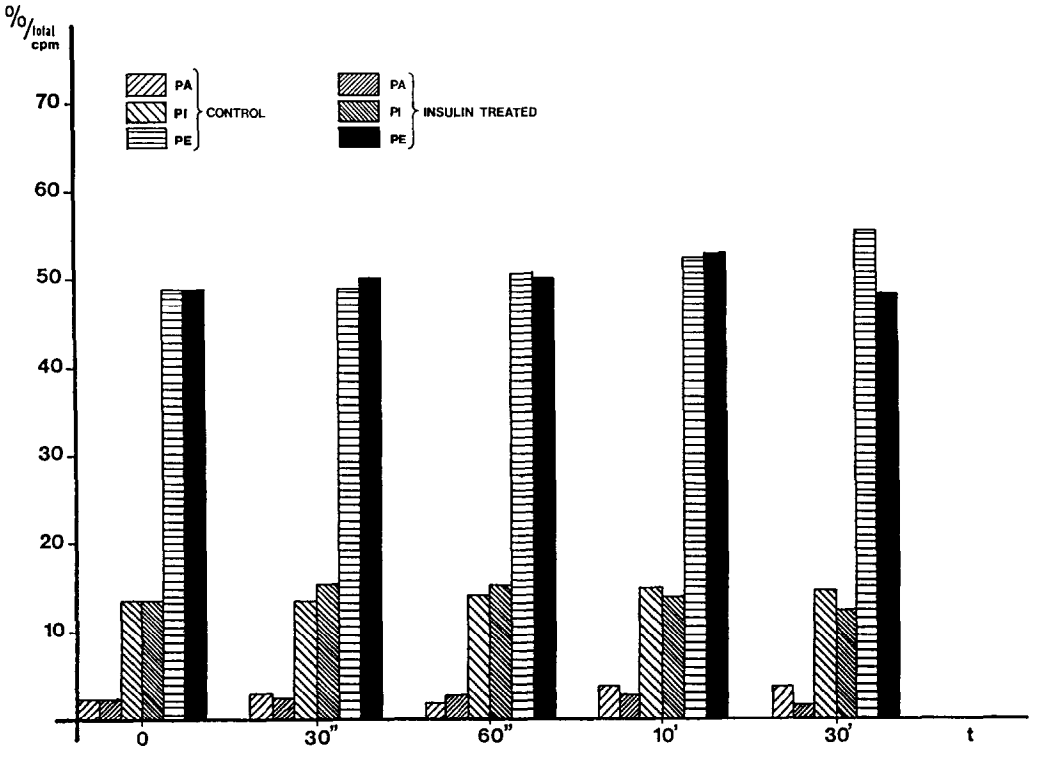

Fig. 2. Phosphatidic acid (PA), phophatidylinositol (PI) and phosphatidyl-ethanolamine (PE) levels in control (not pretreated,not treated) and insulin treated (not pretreated) Tetrahymena.

cells to insulin caused no significant change in the phospholipid values assessed after imprinting (Fig. 3,4).

DISCUSSION

The unicellular Tetrahymena is a suitable model for the experimental study of receptor phylogenesis, and for investigations into the mechanism of hormonal imprinting as well.Indirect evidence was obtained that interference with certain elements of the hypothetical signal transduction system of Tetrahymena also affected the mechanism of hormonal imprinting. This supported the implication that the PI-system, too, was involved in hormonal imprinting,to judge from inhibitability of the latter by neomycin treatment(14).

Hormonal imprinting can durably alter the quantitative relations and activity of the second messenger systems in Tetrahymena. We speculated that if inositolphospholipids also played a role in hormonal imprinting, they would respond to it by a change in in-

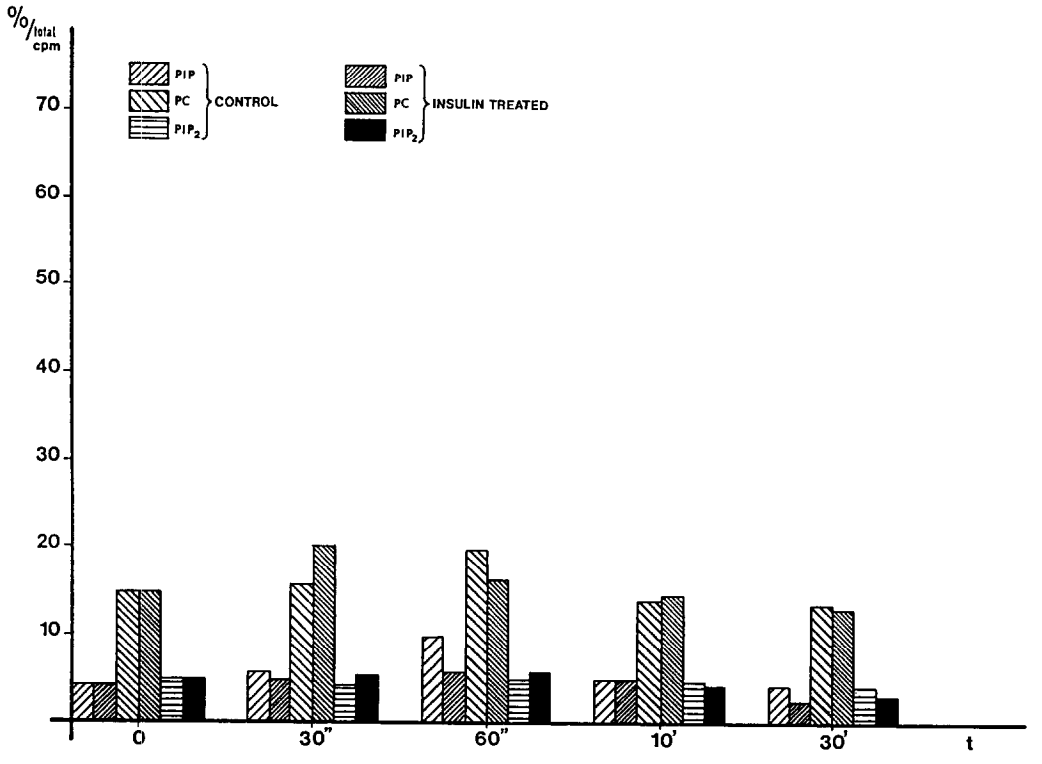

Fig. 3. PIP_2, PIP and phosphatidylcholine levels in pretreated (imprinted) and pretreated and insulin treated Tetrahymena.

tracellular level and/or degradation rate, and evidence of such changes would substantiate the involvement of the PI system in the mechanism of hormonal imprinting.

The receptor-mediated stimulation of cellular functions - which can be elicited by several hormones, neurotransmitters and growth factors - involves among others an increase in the metabolic rate of phosphatidyl-inositol compounds (16). PIP_2, which arises by linkage of membrane-associated PA with inositol, becomes rapidly degraded to inositol tris phosphate (IP3) or diacyglycerol (DG),which initiate as second messengers several important cellular responses (17). Changes in the quantitative relations of these components under the given conditions of experiment presented evidence that the components of the PI-system also occur, and take action, in Tetrahymena.

Pretreatment (primary interaction) with insulin had no appreciable impact on the intracellular levels of the PI components.It follows that the direct effect of the primary interaction with insulin was unrelated to the PI-system not only in higher organisms, but al-

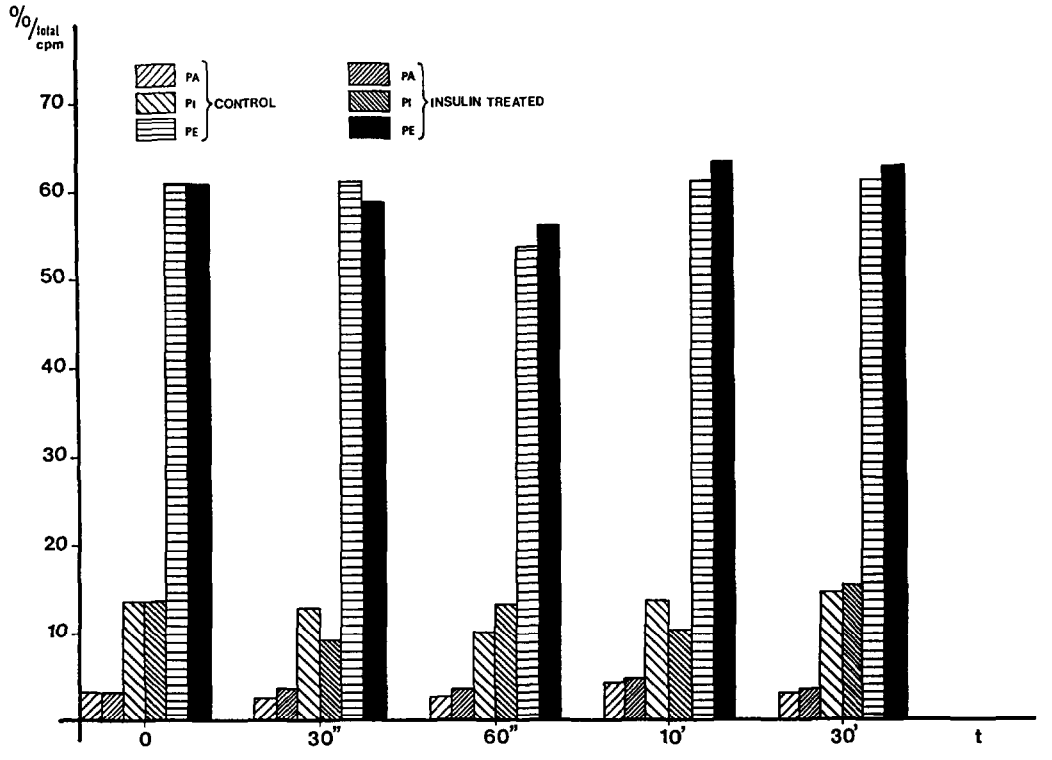

Fig. 4. Phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) levels in pretreated (imprinted)and pretreated and insulin treated Tetrahymena.

so in Tetrahymena. However, 24 h after primary interaction, i.e. in the 6 th to 8th offspring generation of the cells directly involved in hormonal (insulin) imprinting, the intracellular PIP_2 concentration was decreased to 25% of the baseline (control value), and persisted at that level throughout the period of the study. Second exposure to insulin had no impact either on the PIP_2-level.Obviously, not so much the insulin treatment in itself, as the process of imprinting had accounted for the quantitative reduction of the second messenger (PI) components to one quarter of the baseline.In earlier studies we observed a similar adjustment to "pilot light" of another second messenger, the guanylate-cyclase-regulated Ca-calmodulin system, in the conditions of hormonal imprinting(9). Comparison of that finding with the present observations suggests that although imprinting accounted for diminution of the second messenger synthesis, the reduced quantity of second messengers was sufficient for induction of a normal, or even greater cellular response, apparently under an "economy programme"

of the cell. There is reason to postulate that the two "energy saving" second messenger systems were interdependent, because evidence of the association of guanylate-caclase activation with phosphoinositol turnover and Ca^{2+} -mobilisation was already obtained in experimental conditions (18).

The present experimental observations cannot resolve the problem whether the Tetrahymena cells directly involved in primary interaction with insulin were responsible for the changes observed, or transmission of imprinting to several progeny generations accounted for a greater cellular response at lesser investment of energy The fact nevertheless remains that the properties of the mother cell were reproduced in the daughter cells.

Another conspicuous difference between the imprinted and not imprinted cell populations was the difference between their PE levels, which changed similar to PIP_2, but with an opposite character. While insulin had little effect, if any, at the first treatment, a marked PE-elevation followed 24 h later in the offspring generations, which was not appreciably altered by the second insulin exposure. The physiological role of this phenomenon is obscure for the time being. Tetrahymena, although it definitely represents a low level of phylogenesis, is an eukaryotic cell.While the relative proportion of PE is very high (70%) in the phospholipid fraction of bacteria (19), it is comparatively low in mammalian cells (e.g.only 7% in the liver cells). PE was the largest phospholipid fraction also in Tetrahymena,and it showed a further increase under the influence of imprinting.Since the role of PE in membrane viscosity changes is still obscure in many respects, it remains a hypothetical conclusion that it probably accounts for the membrane fluidity required for receptor formation.The fact nevertheless remains that none of the six phospholipid fractions studied changed appreciably immediately after insulin exposure, whereas later PIP_2 and PE equally showed a concentration change, although with an opposite character,as a result of imprinting.

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