Folia biologica (Kraków), vol. **53** (2005), No 3-4

Glucose, Sorbitol and Insulin as Exogenous Agents that Monitor the Interautogamous Interval (IAI) in *Paramecium primaurelia*

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Accepted September 6, 2005

PRAJER M. 2005. Glucose, sorbitol and insulin as exogenous agents that monitor the interautogamous interval in *Paramecium primaurelia*. Folia biol. (Kraków) **53**: 215-221.

Results from previous studies have revealed that the duration of the interautogamous interval (IAI), expressed as the number of fissions occurring between two successive autogamies, is stable and characteristic for species of the *Paramecium aurelia* complex (KOŚCIUSZKO & PRAJER 1988; PRAJER & KOŚCIUSZKO 1999). The duration of the IAI is genetically controlled (KOŚCIUSZKO & PRAJER 1989; MIKAMI & KOIZUMI 1983). Genomic mutations (PRAJER & KOŚCIUSZKO 1999; TAKAGI *et al.* 1989) as well as disturbances of morphological homeostasis of the cell, as observed in *Paramecium* doublets (PRAJER *et al.* 1999), shorten the duration of the IAI. This suggests that epigenetic factors may accelerate the induction of autogamy. The aim of this study was to search for environmental, exogenous factors which may affect the IAI duration. The first investigated factor was glucose used in such a concentration which did not change the rate of vegetative cell divisions. The second tested factor was sorbitol used as nonmetabolized sugar and applied at a similar concentration as glucose to verify a possible osmotic effect. Both these factors accelerated the induction of autogamy and shortened IAI duration in *Paramecium primaurelia.* An insulin hormone was also used as a candidate for a sugar dependent factor complementary to glucose. It also resulted in acceleration of autogamy induction. It was chosen to test its effect on IAI since the existence of an insulin receptor in *Tetrahymena* has been reported (CHRISTENSEN *et al.* 2003). This hormone peptide also induced an IAI shortening. The results of a search in the *Ciliates* genome for potential homologs of genes coding insulin receptors and insulin itself, as well as hypothetical mechanisms of action of investigated agents were discussed.

Key words: Glucose, sorbitol, insulin, meiosis, autogamy, interautogamous interval, *Paramecium primaurelia.*

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Paramecium, an eukaryotic, unicellular organism, with its whole genome sequenced (SPERLING *et al.* 2002; ZAGULSKI *et al.* 2004) and a genome database in the –process of creation, is an excellent model species for the study of mechanisms regulating the processes of development and morphogenesis on both physiological and genetic levels (IFTODE *et al.*1989, 2001; JERKA-DZIADOSZ *et al.* 1992; KACZANOWSKA *et al.* 1996; KRZYWICKA *et al.* 2001; PRAJER *et al.* 1997, 1999; RUIZ *et al.* 2000). It is also exemplary for the elucidation of factors affecting growth and clonal life (HAGA 1991; KOŚCIUSZKO & PRAJER 1989, 1992; MIWA 1984; PRAJER 1994; PRAJER & KOŚCIUSZKO 1994). Eukaryote cells of both unicellular and multicellular organisms have a limited potential to divide even in nutrient rich conditions. Human cells cultured *in vitro* age with progressing divisions and die after a defined number of cell fissions, called the "Hayflick limit" (HAYFLICK & MOORHEAD 1961). Similarly, *Paramecium* cells die after reaching the "Sonneborn limit" of divisions if they do not undergo autogamy, which maintains cell vigor, as was observed in the "Metheuselah" strain cultured for 33 years (SONNEBORN 1954). Thus, the role of sexual reproduction involving meiosis and fertilization is fundamental in preventing ageing and death (TAKAGI 1988, 2000). In the *Paramecium aurelia* species complex two alternative developmental pathways exist, and both of them are induced by starvation and begin at fertilization. One of them leads to conjugation, i.e. the sexual process in which two cells of complementary mating type participate, while the other leads to autogamy, i.e. the self-fertilization process that occurs in the absence of complementary mating type partners. Both sexual processes require maturity to switch from vegetative cell divisions to meiosis. Thus, the beginning of autogamy is under dual control of feeding conditions and the attainment of

autogamous maturity following maturity for conjugation. The period between two successive autogamies is called the interautogamous interval (IAI) (SONNEBORN 1974). Its duration expressed as the number of fissions (SMITH-SONNEBORN & REED 1976), is a stable and characteristic trait for species and even for stocks (KOŚCIUSZKO & PRAJER 1988; PRAJER & KOŚCIUSZKO 1999). This trait is genetically controlled by the macronucleus and can be experimentally accelerated (MIKAMI & KOIZUMI 1983; KOŚCIUSZKO & PRAJER 1989). The isolation of mutants with a short period of autogamous immaturity (TAKAGI *et al.* 1987, 1989) as well as with a long one (KOMORI *et al.* 2004) confirmed the role of the macronucleus in the regulation of IAI duration. The role of the macronucleus and cytoplasm has been widely investigated (for review see PRAJER & KOŚCIUSZKO 1998). It was shown by microtransplantation techniques, that the young, postautogamous cells contain a cytoplasmic factor retarding the expression of a gene(s) inducing autogamy (KOŚCIUSZKO & PRAJER 1992; PRAJER 1994).

Our subsequent approaches focused on the associations of the mechanisms regulating IAI duration with morphogenesis and developmental processes in abnormal forms of *Paramecium,* such as artificially obtained doublets or morphologically affected mutant cells. It was observed that in such types of cells autogamy occurs at a substantially increased rate than in normal, wild cells (PRAJER *et al.* 1999). Therefore, besides nuclear and cytoplasmic factors, some epigenetic stimuli being endogenous factors, such as changes in the structures of the cortex, had an impact on the IAI period and the induction of meiosis preceding self-fertilization.

The aim of this study was to search for environmental, exogenous factors which may affect the IAI duration. The first investigated factor was glucose. It was used in a concentration that did not change the rate of vegetative cell divisions; however it shortened the IAI in *Paramecium primaurelia.* Glucose may affect the cell pathways in different ways. It may induce osmotic stress or it may be metabolized within the cell inducing glucose dependent pathways. Both of these possibilities were verified. Sorbitol was used for the induction of osmotic stress , as a sugar which is not metabolized within the cell and has a similar molecular size as glucose. In cell lines treated with this agent the IAI was also shortened similarly as in the cell lines submitted to glucose action. In this study an insulin hormone was also used as a candidate for a sugar dependent factor complementary to glucose. It was chosen to test its effect on IAI duration since there was a report on the presence of an insulin receptor in *Tetrahymena* (CHRISTENSEN *et al*. 2003). This hormone peptide also induced IAI shortening. Therefore, a search in the *Ciliates* genome for potential homologs of genes coding insulin receptors and insulin itself was undertaken. The *Tetrahymena* genome data base was explored since the *Paramecium* database was only partially available at this moment.

Material and Methods

Standard model species *Paramecium tetraurelia* stock d4-2, and *Paramecium primaurelia* stock 90 (SONNEBORN 1974) were used in this study. *P. primaurelia* was chosen because it has an IAI of long duration (SONNEBORN 1974; KOŚCIUSZKO & PRAJER 1988), giving the opportunity to precisely observe the changes in the length of IAI induced by the tested factors in the culture medium. However, some experimental series were carried out on *P. tetraurelia* characterized by a short IAI. The cells were cultured according to SONNEBORN'S method (1970) in triple depression slides, in 0.4 ml of medium inoculated with *Enterobacter aerogenes,* at 27°C. The medium used in all experiments was slightly expired, however the rate of divisions was comparable with that in freshly prepared medium. The effect of glucose (SIGMA) and sorbitol (SIGMA), both in the same concentration: 1.4 mM was examined simultaneously with that of the entire molecule of bovine insulin (ICN Biomedicals, Inc.) in a 7.2 μM concentration. The effect of the β-chain of bovine insulin (SIGMA) in a 10.0 μM concentration was tested separately.

Autogamy was induced by natural starvation in daily isolation lines according to SONNEBORN'S protocol (1970). Lines, i.e. the clones were always established from autogamous cells. Each day one cell was reisolated into the next slide which was filled with cultured medium (control) or with the experimental one, where it divided a maximum of 7 times. These clones were slightly starved after reaching the stationary phase. Such cells were checked for the occurrence of autogamy using lacto-orcein (BEALE & JURAND 1966) and cultured until mass autogamy (>95%) appeared. The number of fissions was calculated daily, following cell isolation. The sum of fissions, beginning from the day of line origin at autogamy, was taken as the age estimator of the clone (SMITH-SONNEBORN & REED 1976).

Each series of experiments was composed of 12 control cell lines (with the exception of the experimental series performed on *P. tetraurelia,* composed of 36 lines), which were constantly cultured in non-supplemented medium, and a similar number of experimental lines conducted to autogamy si-

multaneously in this medium supplemented with the tested agents.

The Student's *t*-test was used to check if IAIs duration differed significantly between treatments.

Results

The concentrations of glucose and insulin were experimentally chosen and were the maximum concentrations that did not drastically influence the rate of daily divisions, the general outline of the cells, nor the swimming behavior. The concentrations of sorbitol were identical to those of glucose, therefore the osmolarity of the culture medium for both agents was equivalent. In the control, non-supplemented culture medium *P. tetraurelia* as well as *P. primaurelia* cells divided 4-5 times per day. The control and experimental cell lines in each series originated always from autogamous control cells derived from a single clonal line.

As demonstrated in Table 1, the β-chain insulin affects IAI duration neither in *P. tetraurelia* cells nor in *P. primaurelia*. Hence, the presence or absence of this protein in the culture medium at the tested concentration is negligable for the cell fates and for IAI.

In Table 2 the effect of glucose and sorbitol on *P. primaurelia* cells is compared with that of the entire insulin molecule. In contrast to the results presented in Table 1, this substance significantly shortened IAI duration.

Glucose treatment always induced autogamy earlier in comparison with the control series. Moreover, in five series of both agents tested simultaneously, in the medium supplemented with glucose, the mean IAI duration was significantly shorter ($P<0.001$) than in the medium supplemented with insulin.

The effect of sorbitol was tested in five series. The presence of this agent also accelerated the induction of autogamy in the investigated cell clones and significantly shortened IAI duration in com-

Table 1

IAI duration in two *P. aurelia* species subjected to insulin β chain treatment

P. aurelia species	No series	IAI Duration[1]					
		Control			Insulin β-chain treatment 10 μM concentration		
		n	$\overline{X}$	±SD	n	$\overline{X}$	±SD
P. tetraurelia	I	36	18.5	0.52	36	18.75	0.73
P. primaurelia	II	12	56.2	1.28	12	56.0	0.85

n – number of investigated clones; $\overline{X}$ – arithmetic mean of the sample; ±SD – standard deviation; [1] – expressed in number of fissions.

Table 2

Effects of insulin, glucose and sorbitol on IAI duration in *P. primaurelia*

No series	IAI Duration[1]											
	Control			Insulin 7.2 μM			Glucose 1.4 mM			Sorbitol 1.4 mM		
	n	$\overline{X}_C$	±SD	n	$\overline{X}_I$	±SD	n	$\overline{X}_G$	±SD	n	$\overline{X}_S$	±SD
I	12	57.7	3.1	12	34.0	2.2	12	23.0	0.0			
II	12	49.5	6.4	12	30.6	2.3	12	22.7	1.9			
III	12	52.6	4.7	12	41.4	4.6	12	33.0	5.2	12	37.5	2.4
IV	12	50.2	6.6	12	42.2	0.8	12	33.5	3.8	12	37.2	3.2
V	12	6.5	1.2	12	42.0	2.1	12	38.5	2.7	12	40.3	2.7
VI	12	61.5	4.4				12	50.8	1.9	12	48.0	3.5
VII	12	66.2	10.0				12	48.5	1.9	12	51.8	7.2
Total	84	56.4	7.6	60	38.2	5.3	84	35.8	10.8	60	43.0	7.2
Level of significance[3]				$\overline{X}_C$ *versus* $\overline{X}_I$ P<0.001			$\overline{X}_C$ *versus* $\overline{X}_G$ P<0.001			$\overline{X}_C v.\overline{X}_S$ P<0.001		$X_G v.\overline{X}_S$[2] P= 0.2

n – number of investigated clones; $\overline{X}_C$, $\overline{X}_I$, $\overline{X}_G$, $\overline{X}_S$ – arithmetic mean of the sample in the adequate medium; ±SD – standard deviation; [1] – expressed in number of fissions, [2] – series III-VII of glucose and sorbitol treatment were taken into account, [3] – statistically estimated by Student's *t*-test.

parison with the control. The mean IAI duration in the medium supplemented with sorbitol was longer in comparison with the parallel glucose series. However, taking into account five series of cells treated with sorbitol and the respective five series of cells submitted to the action of glucose, the latter difference was statistically insignificant.

The general conclusion is that all investigated agents, with the exception of the β-chain of insulin, significantly accelerated the induction of autogamy. Additionally, glucose reduced IAI duration to a greater extent than the entire insulin molecule. On the other hand, the effect of sorbitol was comparable with that of glucose.

Discussion

According to the presented observations, mild doses of such exogenous agents as glucose, sorbitol and insulin, applied in the culture medium at a concentration that did not affect the course nor the duration of the cell cycle, may accelerate the IAI.

The mechanisms promoting adaptation to variable environmental conditions have been highly conserved during Eukaryotes evolution. Extracellular signals generate a number of different types of cellular responses which are implicated in the control of cell growth and of developmental mechanisms.

Glucose is a source of carbon and energy as an ubiquitous nutrient for eukaryotic cells. It affects many metabolic pathways. Mechanisms involved in glucose sensing are tightly connected with metabolism pathways in animals as well as in plants (ROLLAND *et al.* 2001). In mammalian cells and in the yeasts external glucose in the media induces expression of specific glucose-regulated proteins that may lead to apoptosis (LEE 2001) or inhibit entry into meiosis (HONIGBERG & PURNAPATRE 2003). In contrast, in *Paramecium* cells treated with mild doses of external glucose, an acceleration of the IAI was consistently observed. Since glucose is the agent of both nutritional and osmotic effects, this latter was examined by the use of a non-metabolized sugar, sorbitol. In *Paramecium* cells cultured in medium supplemented with a weak concentration of sorbitol, autogamy occurred more frequently than in control conditions without this agent. Such a result may be interpreted as a case when mild osmotic stress increased the induction of meiosis. This suggests that a mild dose of glucose delivers a simple osmotic shock like sorbitol, rather than a nutritional effect resulting in the inhibition of meiosis. What is the mechanism of autogamy acceleration in *Paramecium* submitted to sorbitol and glucose treatment?

The protein kinases and other messenger systems form integrating networks engaged in control that is based on the existence of different pathways of signal transduction and their interactions. It has been established that the mitogen activated protein kinases (MAPKs), a class of signaling intermediates, perform an essential role in the development of many different types of organisms (PEARSON *et al.* 2001). A subclass of the stress activated protein kinases (SAPK) belongs to the group of MAP kinases, including p38 kinase(s) which responds to osmotic stress stimuli including shocks of hyperosmolarity (MARTIN-BLANCO 2000). This invertebrate p38 kinase has high sequence similarity to the Hog 1 kinase involved in hyperosmotic shock protection in *Saccharoromyces cerevisiae* (PEARCE & HUMPREY 2001). Thus, this kinase family is evolutionary conserved from yeast to mammals. Indeed, WANG *et al.* (1999) found that mild hyperosmotic shock of sorbitol applied to *Tetrahymena* activated the expression of mRNA kinase including TpMAPK which shows significant sequence similarity to the p38 kinases. A different TpNrk also revealed high homology to other NIMA-related protein kinases (WANG *et al.* 1998; NAKASHIMA *et al.* 1999) involved in the control of cell cycle regulation in response to osmotic shock. Thus, the evolutionary conservation of stress activated signaling pathways between mammals and yeast indicates the importance of this regulatory pathway in cellular physiology.

Osmotic stress may induce activation of parallel pathways of the MAP-related kinases in *Paramecium* cells. It is well documented that the stress activated kinases can modulate the course of the cell cycle and affect the checkpoint activation. The mechanisms promoting meiosis in unfavorable conditions are evolutionarily conserved and may resemble the induction of sporulation in the mutant sho1 of yeast (SEET & PAWSON 2004). In these cells with disturbed homeostasis and characterized by diminished function of the mutated scaffold protein Pbs2, high osmolarity shock treatment activated the mating pathways instead of an osmoresponse. One can speculate that *Paramecium,* as *Tetrahymena,* can modify the rate of protein synthesis of MAP kinase cascades as well as signal transduction pathways, and change gene expression in response to various environmental conditions. However, further experiments are needed to confirm this hypothesis.

The presented investigations revealed that entire insulin molecule treatment affects the induction of meiosis and the interautogamous period in *Paramecium* clones. On the other hand, the insulin β-chain had no effect on IAI duration. Insulin, as a

protein hormone, added externally into the medium, stimulates the growth of many types of cells. In *Caenorhabditis elegans* the insulin like signaling pathway controls aging and regulates longevity (TISSENBAUM & RUVKUN 1997). In *Tetrahymena* survival and multiplication depend on nonnutritional cell-produced compounds released to the extracellular fluids or on survival factors such as insulin (CHRISTENSEN 1993; CHRISTENSEN *et al.* 1995). CHRISTENSEN *et al.* (2003) found a protein in the ciliary membranes of *Tetrahymena,* termed *Tetrahymena thermophila* protein tyrosine kinase 1 (TtPTK1), which is tyrosine phosphorylated upon stimulation by the entire insulin molecule as well as by only the insulin β-chain. The sequences of this protein are slightly converged to the *Caenorhabditis elegans* insulin receptor, protein Daf-2, as well as to the NIMA kinase (TpNrk) stimulated by stress (WANG *et al.* 1998; O'CONNELL *et al.* 2003). BLAST searches of the *Tetrahymena* genome primary sequence data revealed numerous genes homologous to the gene of TtPTK1 kinase. Thus, the presence of the ortholog of the gene of TtPTK1 kinase in the *Paramecium* genome was confirmed. However, a search for the trasmembrane insulin receptor-like protein(s) did not confirm the presence of this protein(s) in the *Tetrahymena* genome. A characteristic trait of insulin, according to the pfam classification (http//www.sanger.ac.uk/cgi-bin/Pfam) (SONNHAMMER *et al.* 1998), is the presence of the insulin type domain. This domain exists only in the proteins of the Insulin/IGF/Relaxin family. Among proteins actually known, 339 proteins of *Metazoa* and only one bacterial protein from *Brevibacillus brevis* Q7M0U6-BREBE possessed this domain. This suggests that the presence of the insulin hormone may be limited to *Metazoa*. On the other hand, many publications have described the effects of insulin on *Protozoa* (CSABA & LANTOS 1975; KOHIDAI *et al.* 1992; MUGNAINI *et al.* 1995; SHEMAROVA *et al.* 2002) and showed that insulin is produced in *Tetrahymena* (CSABA *et al.* 1977; CSABA & KOVACS 1995) and other ciliates (LE ROITH *et al.* 1980). Furthermore, the existence of the insulin receptors has been stated (CSABA *et al.* 1992; CSABA & HEGYESI 1994). However, the presence of insulin was demonstrated using biochemical methods exclusively. Since the *Paramecium* genome database is not yet fully accessible, we verified if genes coding for insulin are present in the *Tetrahymena* genome (http://www.tigr.org/tdb) by using bioinformatics methods. In this aim each genome fragment was translated to protein sequences in six ORFs, using the ciliate codon table and the EMBOSS programs (RICE *et al.* 2000). The alignment of 392 sequences of the insulin domain, available at http://www.sanger.ac.uk/cgi-bin/Pfam, was performed to verify the truth/false of this assumption. This domain was applied to the calibrated hidden Markov model (BALDI *et al.* 1994) and the best homologs with expected value <1 were chosen. Then, the BLASTP program was used, which showed that the hypothetic insulin-like peptides were not the homologs of insulin. Thus, one can conclude that in in the *Tetrahymena* genome there are no sequences coding for insulin, although this hormone can affect the life functions of protozoans and these unicellular organisms can produce proteins immunologically similar to insulin. The biochemical and immunological results not confirmed by the bioinformatics methods cannot be considered as proof of the presence of the insulin peptides in *Protozoa.*

It was suggested that insulin pathways exert an effect on lifespan by regulating a variety of genes including the cellular stress response. Some exogenous proteins can nonspecifically stimulate the activation of phagocytosis. Excessive endocytosis can affect cell homeostasis and according to the assumed hypothesis, may induce meiosis. Thus, the entire molecule of insulin in *Paramecium* cells may act as an unspecific inducer of pinocytosis or phagocytosis crucial for the induction of shortening of IAI duration. Therefore the non-receptor mechanism of insulin accumulation may be taken into account in this organism. The endocytotic pathway may be energy independent as evidenced in nuclei of rat hepatoma cells (HARADA *et al.* 1992). The non-receptor trafficking of insulin as passive transcapillary diffusion has also been described in the dog hindlimb (HAMILTON-WESSLER *et al.* 2002). Another speculated hypothesis explaining the effect of insulin on meiosis induction, in spite of a lack of cellular receptors for this protein, is that insulin may mimic the action of some factors present in the natural environment of *Paramecium.*

Acknowledgements

The author expresses its sincere thanks to Dr. Szymon KACZANOWSKI from the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences for his helpful bioinformatics guidance in genome data treatment.

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