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Tetrahymena termophila as a Source of the Opioid Receptors for Testing of Opioid Peptides

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Abstract

Limitations on the use of animals for biological experiments has forced a search for alternative simpler organisms for testing the biological activity of organic compounds. The aim of this research was to use *Tetrahymena termophila* for testing the opioid activity of peptide, β-casomorphin-7, isolated from cheese. Three kinds of test were used: 1) test for inhibition of protozoa mobility, 2) trap test, and 3) phagocytose test. The results for the trap test suggests that it can be used to determine the presence of harmful substances occurring in food. Determination of the opioid properties using the phagocytose test showed that morphine inhibits the phagocytose process of *Tetrahymena termophila*. β-casomorphin-7 had smaller inhibitory effect on phagocytosis than morphine. It can be said that our results and literature prove the possibility of using protozoa *Tetrahymena termophila* for studies of antinutritional substances and opioids in raw and food products.

Keywords: *Tetrahymena termophila*, opioid receptors, opioid peptide, β-casomorphin-7

Introduction

Biological testing of food has become a more important and necessary task. The accomplishment of the task is, however, difficult because of present tendencies to limit studies with animals. Therefore, other more simple organisms should be chosen for testing biologically active compounds in food. The results of studies obtained so far indicate that protozoa, particularly *Tetrahymena,* could be such organisms.

Tetrahymena thermophila protozoa feed on both organisms smaller than they are themselves and on food dissolved in water. Certain substances are distinctly chemoattractive for this species, whereas other substances deter them. This fact allowed using the *Tetrahymena termophila* as indicator organisms in ecotoxicological studies for the evaluation of purity of soil and water. Koppellhus et al. [1] studied the effect of different substances, i.e. bovine blood albumin, insulin, β-endorphin, Met-enkephalin, acetylcholine, and amino acids on the behavour of *Tetrahymena termophila* [1]. This study proved that amino acids and peptides are particularly chemoattractive for these protozoa. The use of *Tetrahymena termophila* in the studies on opioids was possible due to the presence of μ receptors on the outer cell membrane of these organisms [2]. Chiesa et al. [2] characterised the opioid receptors involved in the process of phagocytosis in *Tetrahymena termophila.* The results of this study indicate that morphine and β-endorphin were the most effective agonists of these receptors. Chiesa et al. [2] reported after O'Neill that the opioid receptor of *Tetrahymena termophila* corresponds in respect of biochemical characteristics to μ receptor present in the rat brain. The opioid receptors of *Tetrahymena termophila* participate in phagocytosis and show high sensitivity to naloxone and some opioids, that is close to the sensitivity of μ opioid receptors in the intestine of guinea-pig to the same ligands.

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Koppelhus et al. [1] proposed to accept the opioid receptor of *Tetrahymena termophila* as a model in the studies on structure, function and evolution of opioid receptors. This proposal is supported by the later study by Renaud et al. [3], who proved the participation of protein G in the mechanism by which opioids modulate the phagocytosis in *Tetrahymena termophila* [3]. This mechanism is very much like the one present in more complex organisms. The studies showed that opioid receptor of *Tetrahymena termophila* is a protein with the molecular weight of 110 kDa [10].

The promising results of studies on *Tetrahymena termophila* allow hope that these organisms could be used for biological testing of food and would make a new system of food quality control, complementary to chemical studies.

Experimental Procedure

Tetrahymena termophila protozoa from Nencki Institute of Experimental Biology in Warsaw were used for biological studies on opioid peptide, β-casomorphin-7, isolated from Brie cheese. β-casomorphin-7 from Sigma (St. Louis, MO, USA), β-casomorphin-7 isolated from Brie cheese, and morphine from Polfa were used in the study. The culturing of protozoa was carried out in axemic conditions (without the presence of other organisms) on a standard medium PPY composed of 0.7% peptone (Sigma), and 0.7% yeast extract (Sigma). The protozoa were sampled for experiments at the phase of logarithmic growth.

The Procedures for Pharmacological Tests of Opioids with the Use of *Tetrahymena termophila* [2, 1, 5]

1. Test for inhibition of protozoa mobility: An aliquot (0.1 cm^3) of examined solution (morphine, β-casomorphin-7 from Sigma, naloxone,) and 0.01 cm^3 of *Tetrahymena termophila* suspension were placed in wells of a titration plate used for the ELISA test. The number of protozoa whose mobility was inhibited was calculated under a microscope (Biopolar) at 50x magnification after 10 and 60 min. The results were expressed as percentages, assuming the number of protozoa observed in the microscope visual field to be 100%. The average number of protozoa observed in the visual field was 50±5. The experiments were repeated 3 times.

2. Trap test: This test was carried out with a trap with 12 capillaries. The capillaries filled with examined solutions, i.e. morphine, β-casomorphin-7 from Sigma, naloxone or water were inserted into a central vessel. Deionized water was used as a reference sample. The central vessel of a trap was filled with suspension of protozoa in deionized water. The whole system was placed in a thermostat at 297K. After 45 min, the capillaries were disconnected from the central vessel and their content was transferred onto microscopic slides. So prepared preparation was fixed with osmium tetraoxide vapours. The number of protozoa was calculated in the visual field of the microscope. The total number of cells was assessed by microscopic image analysis using Multi Scan v. 4.1 apparatus. The experiments were repeated 4 times.

3. Phagocytose test: This test was used for determining the ability of opioid compounds to inhibit the formation of alimentary vacuoles. The dependence of the percentage inhibition degree of *Tetrahymena termophila* phagocytosis by morphine, β-casomorphin-7 (from Sigma) and β-casomorphin-7 isolated from Brie cheese was determined using fluorescing latex balls 0.48 μm (Polyscience). The opioid compounds were prepared at the concentrations of 20, 40, 100 and 200 nM, to which 0.01 cm^3 of 0.025% latex suspension per 0.100 cm^3 was added. To 0.1 cm^3 of so prepared solutions, the suspension of *Tetrahymena termophila* was added and the sample was left for 10 min in thermostat at 297K. To the incubated mixture, 0.01 cm^3 of 2.5% osmium tetraoxide solution was added in order to fix the preparation. The fixed protozoa were washed four times with deionized water to remove the excess of latex. The microscopic preparations were prepared from the fixed protozoa. The number of vacuoles with fluorescing latex was measured in every protozoa by microscopic method in phase contrast (Aksiskop from Opton). The number of alimentary vacuoles was counted in tens of protozoa and the arithmetic mean for three repetitions (± SD) and was calculated. The obtained results were analysed statistically with Student's t-test. The number of vacuoles per one protozoa was converted into per cent of phagocytosis inhibition according to the formula:

$$\% \text{ phagocytosis inhibition} = 100 - (\text{Ph.F.} \times 100),$$

where Ph.F. is phagocytosis coefficient that expresses the ratio of vacuoles in protozoa incubated with the examined compounds to the number of vacuoles in the control sample with deionized water. The curves for per cent inhibition of phagocytosis in *Tetrahymena termophila* depending on the concentration of examined opioid agonists were obtained by computer analysis and Grapher program (Golden Softwer USA). These data allowed to determine maximal dose (E_{max}) and effective dose (ED_{50}) of agonist concentration causing the phagocytosis inhibition as follows: 100 and 50%. The E_{max} values were used for determining the relative activity of examined opioid compounds:

$$\alpha = E_{max} \text{ tasted opioid}/E_{max} \text{ morphine}$$

4. Isolation of β-casomorphin-7 from Brie cheese [7]. β-casomorphin-7 was extracted from Brie cheese as follows: ground cheese was homogenised with chloroform/methanol mixture (2:1 v/v).

The methanol fraction was evaporated and lyophilised. The peptide extract was successively separated by following chromatographic methods: thin layer chromatography on silica gel, Sephadex G-50 gel filtration, chromatography on SP Sephadex G-25. The sequence of β-casomorphin-7 was determined on the basis of amino acid composition

and minimal mole ratios and on the identity of the N- and C-terminal amino acids and comparision of these date with the sequence of the milk β-casein. To confirm the structure of this peptide, its opioid activity was determined.

Results

The results of the test for inhibition of *Tetrahymena thermophila* mobility are presented in Table 1. The observation of protozoa mobility in the solutions of examined substances allowed to state that naloxone at concentration $1x10^{-3}$ M had the strongest inhibitory effect, which was four times and about fifteen times stronger than the effects of morphine and β-casomorphin-7, respectively. The results presented in Table 1 correspond to the observations carried out for 10 min.

Protozoa undertook their normal motor activity after 60 min. This test was not subjected to deeper analyses because of unsatisfactory specificity and difficulties with the method standardization. It is interesting to note that as high concentration of morphine as 1×10^{-3} M does not kill protozoa. The results obtained in the trap test are presented in Table 2.

Our study has proved that the alluring properties of *Tetrahymena termophila* against morphine and β-casomorphin-7 solutions were significantly different ($p \leq 0.05$) than against their antagonist, naloxone. β-casomorphin-7 had slightly stronger alluring effect against protozoa than water. On the other hand, the action of naloxone was strongly deterrent in comparison with morphine.

The results obtained by the phagocytosis test are compiled in Table 3. Figure 1 presents the percentage of inhibition degree of *Tetrahymena termophila* phagocythosis by morphine, β-casomorphin-7 (from Sigma) and β-casomorphin-7 isolated from Brie cheese. The phagocytose test shows that the changes in the number of nutrition vacuola in *Tetrahymena termophila* cased by the opioid were statistically significant only up to the concentration of 100 nM ($p \geq 0.05$). The influence of the naloxone-morphine mixture (in ratio 1:1 w/w) on *Tetrahymena termophila* phagocytosis is presented in Table 5. The highest inhibition of *Tetrahymena termophila* pagocytosis

Table 1. Results of the test for inhibition of *Tetrahymena termophila* mobility.

Substance	% inhibition at concentration 1 x 10^{-3}M (a) (mean ± SD)	% inhibition at concentration 1 x 10^{-6}M (a) (mean ± SD)
Water	0.0 (a)	0.0 (c)
Morphine	15.3 ± 2.49 (a) (b)	8.8 ± 1.88 (c) (d)
ß-casomorphin-7 (Sigma)	4.0 ± 0.81 (a) (b)	2.6 ± 0.63 (c) (d)
Naloxone	57.3 ± 6.17 (a) (b)	10.0 ± 1.63 (c) (d)

(a) Probability level = 0.95; (b) For three determinations, a, b, c, and d - statistically significant differences

Table 2. Effect of substances with opioid activity on the number of cells in traps.

Substance	Average number (± SD) of cells in trap
Water	1670 ± 75 (a)
Morphine at 1 x 10^{-3} M concentration	1309 ± 73 (a) (b)
ß-Casomorphin-7 at 1 x 10^{-3} M concentration	1728 ± 169.8 (a)
Naloxone at 1 x 10^{-3} M concentration	282 ± 31.21 (a) (b)

Probality level = 0.95; For three determinations; a, b - statistically significant differences

Table 3. Dose-dependent effects of morphine and β-casomorphin-7 (purchased from Sigma and isolated from cheese) on latex phagocytosis by *Tetrahymena termophila.*

Concentration of tested opioids	Morphine		ß-cosomorphin-7 (Sigma)		ß-cosomorphin-7 (Cheese)	
	Vacuola number per cell (mean±SD)	%inhibition (mean±SD)	Vacuola number per cell (mean±SD)	%inhibition (mean±SD)	Vacuola number per cell (mean±SD)	%inhibition (mean±SD)
0.0 nM(water)	4.43 ± 0.29 (n = 46)	0.0 (a)	4.43 ± 0.29 (n = 46)	0.0 (c)	4.43 ± 0.29 (n = 46)	0.0 (e)
20 nM	3.69 ± 0.33 (n = 53)	16.52 ± 1.48 (a) (b)	3.71 ± 0.23 (n = 52)	16.3 ± 1.01 (c) (d)	3.81 ± 0.31 (n = 29)	13.80 ± 1.12 (e)
40 nM	3.20 ± 0.28 (n = 49)	27.60 ± 2.41 (a) (b)	3.41 ± 0.53 (n = 29)	23.10 ± 3.59 (c) (d)	3.79 ± 0.30 (n = 27)	14.26 ± 1.12 (e) (f)
100 nM	2.21 ± 0.36 (n = 32)	50.23 ± 8.41 (a) (b)	3.20 ± 0.25 (n = 73)	27.80 ± 2.17 (a) (d)	3.17 ± 0.33 (n = 51)	28.28±2.94 (e) (f)
200 nM	2.00 ± 0.35 (n = 52)	54.77 ± 9.35 (a) (b)	3.00 ± 0.23 (n = 59)	32.30 ± 2.47 (c) (d)	3.05 ± 0.27 (n = 36)	31.00 ± 2.75 (e) (f)

Probality level = 0.95; For three determinations; a, b,c,d,e and f - statistically significant differences

Table 4. Concentration values for ED_{50}, E_{max} and relative activity α.

Substance	ED_{50}	E_{max}	α
Morphine	34	250	1.00
ß-casomorphin-7 standard	20	220	0.88
ß-casomorphin-7 isolated from cheese	24	200	0.80

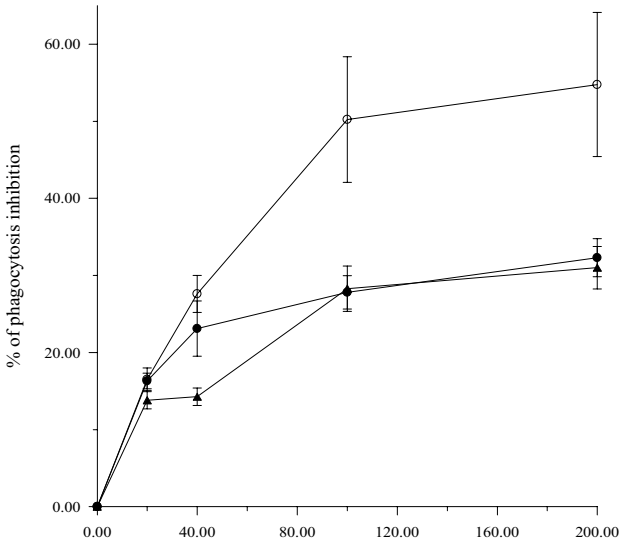

Fig. 1. Curves for inhibition of latex phagocytosis depending on the concentration of opioid substances: morphine (○), β-casomorphin-7 (from Sigma) (●), β-casomorphin-7 (from Brie cheese) (▲).

was observed at the concentration 1 x 10^{-8} M of the naloxone-morphine mixture. The increase in the concentration of this mixture up to 1 x 10^{-4} M devreased the inhibition of *Tetrahymena termophila* phagocytosis to 26% of that at the concentration 1 x 10^{-8}.

The shape of the curve suggests that β-casomorphin-7 is a less active and less effective agonist than morphine. The assessment of this fact in numbers is given by coefficient α. The activity of morphine in the interaction with opioid receptors of *Tetrahymena termophila* is about 1.7 times greater than that of β-casomorphin-7. The influence of examined substances on *Tetrahymena termophila* phagocytosis is illustrated by Fig. 2.

Discussion of Results

Milk proteins are a rich source of peptides that show opioid properties. Opioid peptides have also been isolated from the samples of some commercial rennet and mould cheeses [7, 8, 9]. The present state of knowledge does not allow to answer the question of what is the physiologically safe level of β-casomorphin-7 in the mammal organism. In healthy people there is found the presence of opioid peptides in the small intestine after intake of milk [10]. It is not known whether the *ad libitum* intake of dairy products may be a reason for diseases. In this context the biological testing of food is necessary.

Tetrahymena termophila is an example of a lower organism which potentially can be used to test the opioid peptides derived from food proteins. This statement requires a somewhat broader comment. Protozoa which do not have a nervous system use the language of chemical signals to be oriented in an environment. The surface receptors of *Tetrahymena termophila* allow it, first of all, to find food and to keep out of danger. It has been found that *Tetrahymena termophila* protozoa have μ receptors (opioid receptors) on their surface, of which affinity to morphine and naloxone is very similar to the affinity of

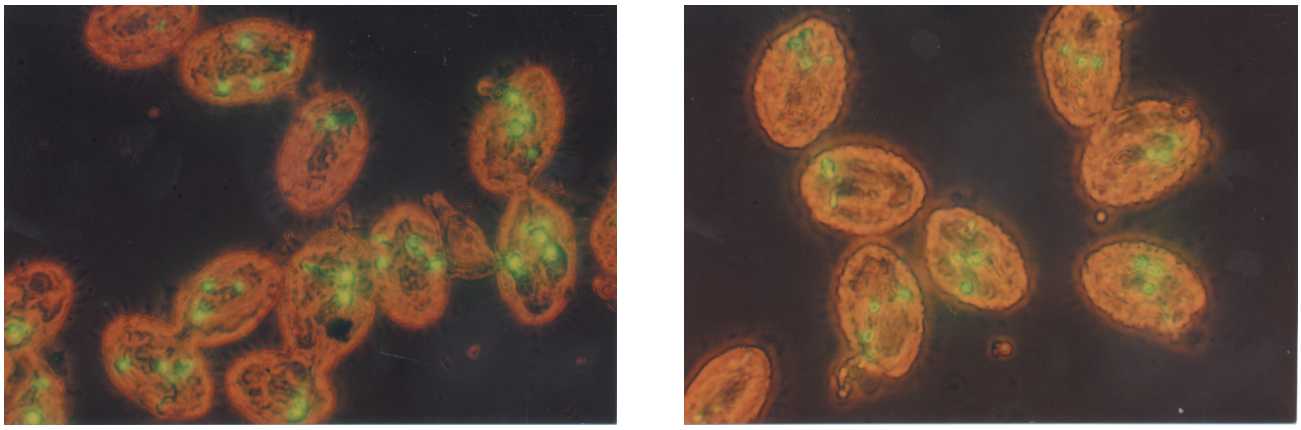

Fig. 2. Influence of examined opioid substances on *Tetrahymena termophila* phagocytosis.

Table 5. Influence of naloxone-morphine mixture (in ratio 1:1 w/w) on *Tetrahymena termophila* phagocytosis.

Concentration of naloxone-morphine mixture (in ratio 1:1 w/w)	Vacuola number per cell (mean ± SD)	%Inhibition
water	4.43 ± 0.29 (n = 46)	0.0 (a)
1 x 10^{-8} M	3.10 ± 0.29 (n = 59)	50 (a) (b)
1 x 10^{-7} M	3.66 ± 0.44 (n = 54)	18 (a) (b)
1 x 10^{-6} M	3.78 ± 0.32 (n = 55)	17 (a)
1 x 10^{-5} M	3.62 ± 0.30 (n = 79)	15 (a)
1 x 10^{-4} M	2.22 ± 0.44 (n = 53)	13 (a)

Probability level = 0.95; For three determinations; a, b – statistically significant differences

receptors present on snail phagocytose cells and in guinea pig small intestine [12]. In addition, studies pertaining to the mechanism of intracellular signal transmission following the receptor stimulation in protozoa showed that membrane G proteins are engaged in this process, so the mechanism corresponds to the one working in higher animals [2, 5]. Opioid receptor in *Tetrahymena thermophila* is a protein of the molecular weight 110 kDa [10]. Protozoa are easily cultured in a defined medium in axenic conditions. Testing of the opioid activity of food with *Tetrahymena termophila* seems to be easier than with other animals regarding feeding that is much simpler for the former. Moreover, the testing can be made in large number of the repetitions and at a considerably reduced cost. The results obtainable from the studies with protozoa may in some cases be different from those obtainable with higher animals in which various types of opioid receptors may be engaged in one reaction at the same time. Free amino acids and medium molecular weight peptides are the stimulus for protozoa that increase their mobile and phagocytolytic activities since they are alimentary signal. The studies on different opioids with a peptide character and the results presented in this article indicate a strong affinity of compounds of this type towards the μ receptor . This fact allows the use of *Tetrahymena termophila* in studies on opioids.

The test for inhibition of protozoa mobility (10^{-3} M) and the test for trap showed that naloxone has the strongest inhibitory effect. These results prove that naloxone, as the antagonist, binds stronger to the opioid receptor of *Tetrahymena termophila* than morphine, as the agonist. A partial confirmation of this fact can be the results of the influence of naloxone-morphine mixture (Table 5) on *Tetrahymena termophila* phagocytosis. These results show that together with an increase in the concentration of the naloxone-morphine mixture, the inhibition of *Tetrahymena termophila* phagocytosis decreases. This result seems to confirm the above statement that naloxone binds more effectively to the opioid receptor of *Tetrahymena termophila* than morphine. The Renaud et al. [3] studies showed that permanent exposure of protozoa to opioids can cause a development of tolerance towards inhibitory action of the agonist. This suggests that the state related to narcotic dependence is formed in the protozoa cells. Naloxone affects the addictive cells as a partial agonist [11].

Determination of the opioid properties using the phagocytose test showed that morphine inhibits the phagocytosis process when affecting the opioid receptors of *Tetrahymena termophila*. β-casomorphine-7 had slightly smaller inhibitory effect on phagocytosis in comparison to morphine. Our earlier experiments showed that naloxone, the compound with antagonistic properties, inhibited the phagocytosis process in similar degree as morphine [7]. In this work the equivalent mixture of naloxone-morphine was used at different concentrations to investigate the phagocytosis process in *Tetrahymena termophila*. The results proved that the degree of the phagocytosis inhibition was dependent on the concentration of the naloxone-morphine mixture. Comparing the results obtained in this work with those of our earlier investigations we can state that the effectiveness of the phagocytosis inhibition was smaller in the case of naloxone-morphine mixture than naloxone and morphine when used separately [7]. This fact suggests that naloxone and/or morphine combines competitively with μ opioid receptors.

In summary, it can be said that the results for the conducted experiments prove the possibility of using protozoa *Tetrahymena termophila* for studies of opioid activity of food components. This fact seems very interesting and important, particulary in the aspect of possibly limiting the use of the animals for such investigations. This matter is of ethical and economical significance.

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