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Cent. Eur. J. Biol. • 4(3) • 2009 • 327–334
DOI: 10.2478/s11535-009-0023-3

Central European Journal of **Biology**

Obestatin as contractile mediator of excised frog heart

Research Article

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Received 31 January 2009; Accepted 22 May 2009

Abstract: The aim of this study is to investigate the mechanism of positive inotropic effect of obestatin on *in vitro* heart preparations of *Rana ridibunda* frog. The application of increasing amounts of obestatin in the concentration range from 1 nmol/l to 1 μmol/l significantly enhances the force of contraction of excised and cannulated frog hearts. This effect was partially reduced in the presence of prazosin (3 μmol/l). Propranolol (30 μmol/l), pertussis toxin (2 ng/ml) and the specific inhibitor of cAMP-dependent protein kinase (PKA) Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine (30 μmol/l) completely blocked the obestatin-induced increase of the force of frog heart contractions. It is concluded that, *via* its receptor molecule, obestatin activates neuronal pertussis toxin sensitive G-protein(s) that further enhance the secretion of epinephrine from sympathetic neurons. This epinephrine activates mainly the myocardial β-adrenoreceptors and PKA downstream targets, and is responsible for the observed positive inotropic effect of obestatin. An alternative explanation of our data is that obestatin directly enhances the effect of myocardial β-adrenergic signaling.

Keywords: *Obestatin • Cardiac • Hormone • Autonomic nervous system • In vitro • G-protein*

Abbreviations

GPR39 - G-protein-coupled receptor-39;
PKA - cAMP-dependent protein kinase;
PTX - pertussis toxin;
Rp-cAMPS - Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine.

1. Introduction

Posttranslational modification of preproghrelin, a polypeptide containing 117 residues that is encoded by the ghrelin gene is predicted to yield two peptides with regulatory functions – ghrelin and obestatin [1]. The ghrelin gene is expressed in stomach X/A-like oxyntic gland cells and to a lesser extent in the intestine, placenta, kidney, hypothalamus, pituitary gland, vascular endothelium [2-4] and others. Zhang *et al.* [1] reported that obestatin binds to orphan G-protein-coupled receptor-39 (GPR39). Obestatin-GPR39 binding was

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confirmed in gastrointestinal and adipose tissues [5] but other authors still had doubts about such an interaction (for review see [6]). Recently, significant progress in the understanding of obestatin signaling has been made. It was revealed that obestatin may consecutively activate G_i-protein, phosphoinositide 3-kinase, protein kinase Cε, Src kinase and extracellular signal-regulated kinases [7] as well as β-arrestin and downstream Akt *via* a G_i-protein independent pathway [8] in gastric cancer cells.

Frog heart preparations are very useful for pharmacological studies. *In vivo* measurements of frog heart contractions allow the investigation of complicated interactions of mediators with impact on different endocrine and neuronal influences throughout the body [9]. On the other hand, excised frog heart consists mainly of cardiac muscle tissue with its endothelium and the projections of autonomic neurons. Thus, any observed effect is mediated only by these tissues and therefore, the *in vitro* frog heart preparations are very suitable for studying the mechanism of regulation of transmitters or pharmacological agents that influence the myocardium either directly or indirectly *via* regulatory neurons. The axon projections of these neurons form long chains of varicosities that are seldom in close contact with the cardiac muscle cells of amphibian heart [10]. Acetylcholine- and muscarine-sensitive K^+ channels, as well as adrenergic receptors and functionally related Na^+ and Ca^{2+} channels, are equally and essentially uniformly distributed over the entire surface of the cardiac muscle membrane [10-12] and are often targeted by different regulators and modulators [9].

The aim of this study is to investigate the mechanism of positive inotropic effect of obestatin in heart preparations of *Rana ridibunda* frog *in vitro*.

2. Experimental Procedures

2.1 Subjects

All experimental procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Bulgarian Center for Bioethics. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the Institutional Animal Care and Use Committee, April 1997, Oakland University, USA.

2.2 Frog heart preparation *in vitro*

All experiments were performed at room temperature (20-22°C). Frogs were placed in a bell jar with anesthetic-soaked cotton (ethyl ether). Frogs were killed by double pithing and their hearts were excised and cannulated. The cannula was passed *via* the left aortic branch (*truncus arteriosus sinister*) and aortic trunk (*conus arteriosus*), and then was inserted into the ventricle. The volume of the cannula is approximately 500 µl. Afterwards, their hearts were connected to a force transducer. Contractions were recorded and analyzed on a computer using interface and TENZO1 software (Stocks, Sofia, Bulgaria). Separate time control measurements were performed with the addition of Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine (Rp-cAMPS), propranolol, pertussis toxin and prazosin.

2.3 Solutions and drugs

All substances were dissolved in modified Ringer solution, hereafter referred to as Ringer solution, with the following composition - 100 mmol/l NaCl, 1.3 mmol/l KCl, 0.7 mmol/l $CaCl_2$ and 1.2 mmol/l $NaHCO_3$. This solution (200 µl) was introduced by a cannula into the frog heart aorta. The injections of pure or inhibitor(s)-containing Ringer solution were performed regularly at 15 min intervals during 90 min experiments as presented in Figure 1. Pertussis toxin (holotoxin, PTX) was injected as inactive precursor. Obestatin was applied 30 min after PTX application to allow the entry of the enzymatic component of the holotoxin – the PTX A protomer – into the target cells. The sources of chemicals used were as follows: obestatin - from Bachem AG, Bubendorf, Switzerland; pertussis toxin – from Calbiochem, EMD Biosciences Inc., La Jolla, CA, USA; prazosin, propranolol, Rp-cAMPS and all salts were from Sigma-Aldrich Inc., St. Louis, MO, USA.

2.4 Statistical analysis

All data are presented as means ± SEM. The *n* in the text refers to the number of frog heart preparations *in vitro*. Statistical significance was determined by Student's *t* test for independent samples. $P<0.05$ was considered statistically significant.

3. Results

Frog heart preparations *in vitro* develop regular contractions with stable pattern and force. That is why these preparations are used in various physiological and pharmacological studies, including those of newly discovered transmitter molecules. Under our experimental conditions the spontaneous contractions of excised frog hearts slightly declined during the experiment, preceded by an initial moderate decrease

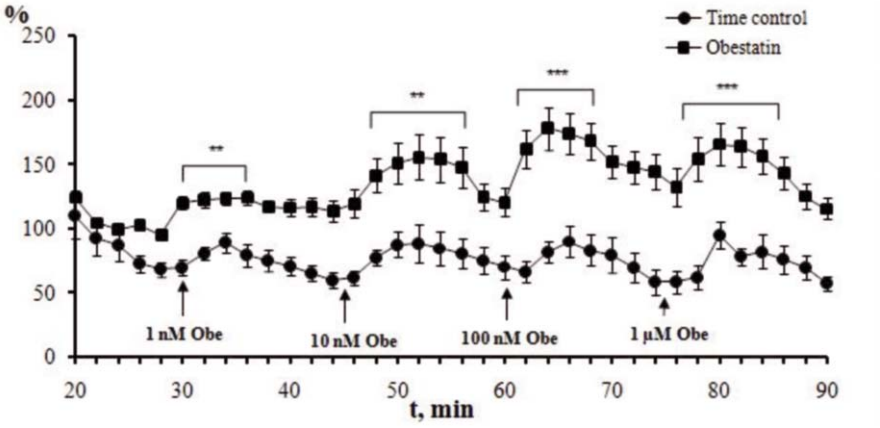

Figure 1. Effect of obestatin on the maximal force of contractions of frog heart preparations *in vitro*. Time course of spontaneous frog heart contractions (●), expressed as percentages of the initial contractile force, measured 10 min from the start of the experiment (taken as 100%), and prior to obestatin administration. The second curve (■) presents the maximal force of contractions after the injection of increasing concentrations of obestatin. Data are means ± SEM of 6 experiments. *** $P < 0.001$ and ** $P < 0.01$ *vs.* time control.

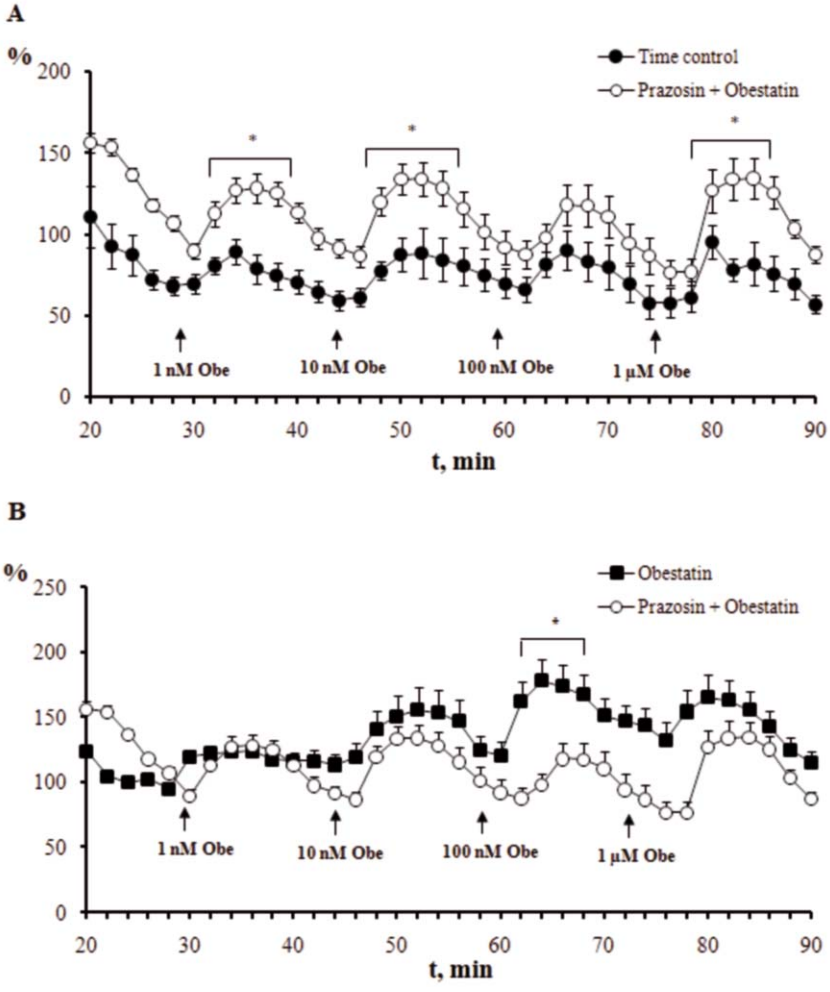

Figure 2. Effect of obestatin added in the presence of prazosin on the maximal force of contractions of the frog heart. Time course and conditions of the experiments are the same as in Figure 1. Prazosin was administered 15 min before obestatin concentrations (○). Time control data (A, ●), as well as the data showing the effect of obestatin alone (B, ■) are given for comparison. Data are means ± SEM of 6 experiments. * $P < 0.05$ *vs.* time control in (A), and *vs.* obestatin + prazosin in (B).

of the force lasting during the first 15-20 min. Therefore, we decided to apply obestatin half an hour after the start of the experiment. Inhibitors were introduced 15 min before the first concentration of obestatin.

We applied increasing concentrations of obestatin such that doses of 1, 10, 100 and 1000 nmol/l obestatin were introduced with a 15 min period between each (Figure 1). The concentration of ghrelin, the other active peptide obtained by ghrelin gene expression is estimated to be about 1 nmol/l in human blood plasma when both the acylated and deacylated forms of the peptide are calculated [13]. Therefore we expected a selective influence on the still unknown obestatin receptor in its lower concentrations. On the other hand, higher doses of 100 nmol/l and 1000 nmol/l guarantee the full effect of the studied transmitter and thus a maximal response of the target tissue, that ensures optimal conditions for cell signaling investigation.

The positive inotropic effect of obestatin was slightly suppressed by 3 μmol/l prazosin, a selective peripheral α_1-adrenoreceptor blocker that was introduced with 200 μl Ringer solution 15 min before the application of obestatin and prazosin together (Figure 2). When compared to the time control only the combination of 100 nmol/l obestatin and 3 μmol/l prazosin increased the force of contractions insignificantly (Figure 2A) while, in the presence of prazosin, the other three concentrations of obestatin (1, 10 and 1000 nmol/l) demonstrated positive inotropic effect similar to obestatin application (Figure 2B).

Further, results suggested that the effect of obestatin on the frog heart preparations *in vitro* was mediated by epinephrine, released as a neurotransmitter that acts mainly *via* β-adrenergic receptors. The effect of obestatin on the heart force of contraction was completely inhibited by pretreatment with 30 μmol/l propranolol, a non-selective blocker of β-adrenergic receptors (Figure 3A). At this concentration, propranolol not only removed the effect of obestatin, but the amplitudes of the force of contraction were lower than the time control values, suggesting a continuous influence of catecholamine release during the whole experiment (Figure 3A). The data of the combined effect of propranolol and obestatin *vs*. those of obestatin presented the largest significant difference ($P<0.001$) within the whole concentration range of obestatin.

Next we tested the participation of PKA using a specific PKA-inhibitor Rp-cAMPS (30 μmol/l). In the presence of this PKA-inhibitor, the effect of increasing concentrations of obestatin on the force of contraction was practically indistinguishable from the time control as illustrated on Figure 3B, *i.e.* the obestatin effect was completely blocked by Rp-cAMPS. For that reason, the inotropic effect of obestatin was significantly different from that obtained in the presence of the mixture Rp-cAMPS and obestatin in the whole range of obestatin concentrations (Figure 3B, squares).

Further, we used pertussis toxin to study the participation of $G_{i/o}$-proteins in the observed effect of obestatin. Pertussis toxin (2 ng/ml), applied 30 min before obestatin, did not allow the studied peptide to increase significantly the control values of the force of contractions (Figure 4, open squares). Thus, when comparing the amplitudes obtained in obestatin-containing modified Ringer solution *vs*. one containing obestatin and pertussis toxin, a significant difference was observed at obestatin concentrations above 1 nmol/l. The effect of obestatin alone is added in this figure for comparison (closed squares).

4. Discussion

The knowledge about the mechanisms of obestatin-dependent regulation of physiological functions is still incomplete. Furthermore, little is known about the effect of obestatin on heart muscle. We aimed to study the mechanism of obestatin regulation of frog heart contractions using the positive inotropic effect of obestatin as a sensor for signaling.

Signaling by cardiac β-adrenergic receptors has been studied in great detail (for review see [14]). The main pathway includes G_s-protein dependent stimulation of adenylate cyclase that increases the cAMP level and thus activates PKA. PKA further phosphorylates several functionally essential cardiac proteins like L-type Ca^{2+} channel [15], ryanodine receptor [16], phospholamban, a regulatory protein of sarco-endoplasmic reticulum Ca^{2+}-ATPase [17], troponin I [18] and others. Frog heart lack functionally significant Ca^{2+}-release stores [19] and for that reason ryanodine receptors and phospholamban will not be of physiological importance. Na^+-Ca^{2+} exchanger in frog hearts works mainly in a reverse mode as a Ca^{2+}-influx mechanism and is inhibited by PKA [20]. Thus, the inhibition of Na^+-Ca^{2+} exchanger by β-adrenergic receptor/adenylate cyclase/PKA pathway will counteract the maximal amplitude of contraction. Our result for a PKA-blockable positive inotropic effect of obestatin cannot be explained with the inhibition of the Na^+-Ca^{2+} exchanger. Therefore, the observed obestatin-induced β-adrenergic receptor stimulation increases the force of frog heart contraction by other mechanisms, most probably by a PKA-dependent activation of L-type Ca^{2+}-channels [13] and/or of regulatory proteins like protein phosphatase inhibitor-1 [21], troponin I [18] and myosin binding protein-C [22].

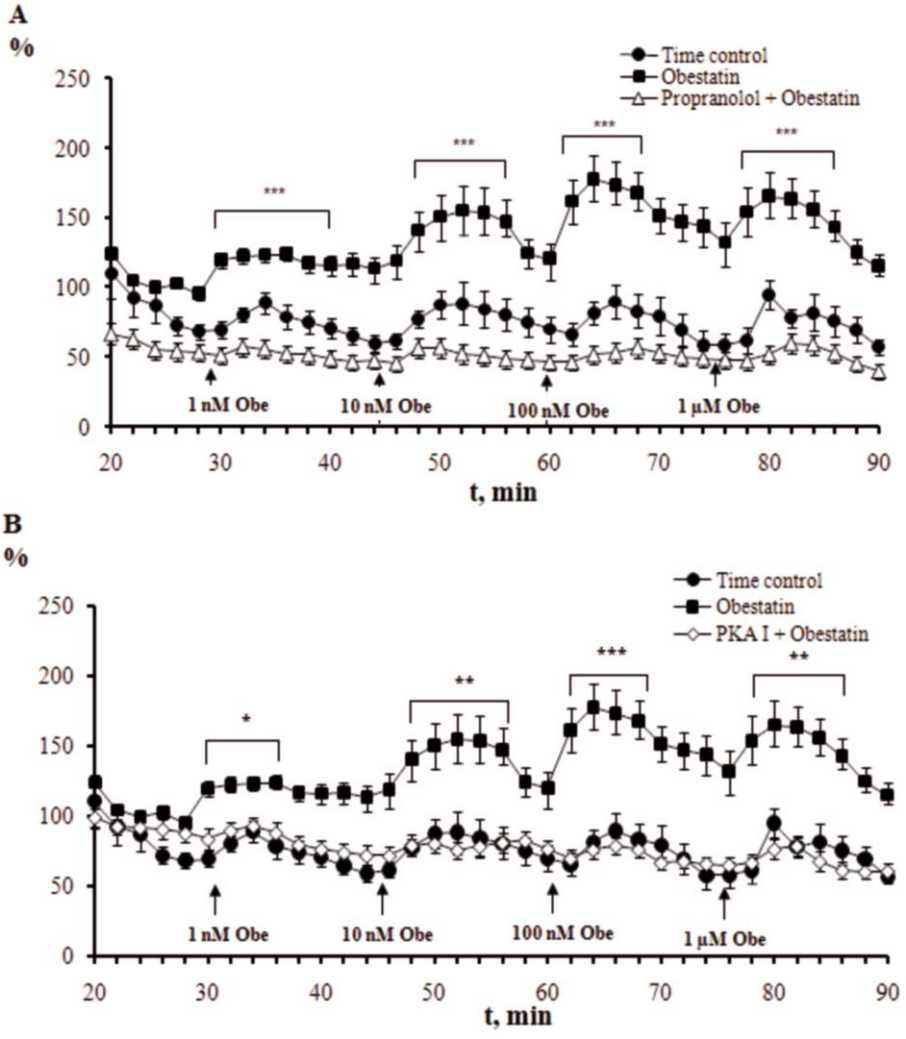

Figure 3. Effect of obestatin added in the presence of propranolol or Rp-cAMPS on the maximal force of contractions of the frog heart. Time course and conditions of the experiments are the same as in Figure 1A. Experiments of frog heart preparations with 30 μM propranolol and application of increasing concentrations of obestatin are illustrated with (△). Time control (●) and obestatin (■) measurements of frog heart preparations *in vitro* are given for comparison. B) Rp-cAMPS (PKA I) 30 μM was injected 30 min before the start of obestatin administration (◊). Time control of maximal contractions (●) and obestatin contractions (■) are given for comparison. Data are means ± SEM of 6 experiments in each (A) and (B). ***P < 0.001 *vs.* obestatin + propranolol in (A); *** P < 0.001, ** P < 0.01 and * P < 0.05 *vs.* obestatin + PKA I in (B).

On the other hand, the small impact of α_1-adrenergic receptors is notable. In the presence of prazosin, the positive inotropic effect of obestatin is only slightly suppressed, which differs from data on frog heart preparations *in vivo* of the same species where corticosteroids potentiate the myocardial α_1-adrenoreceptors to perform a strong positive inotropic effect [9]. A participation of a complex obestatin receptor/β-adrenergic receptor potentiation could explain our data. Thus, in esophageal cancer cells, obestatin induces β-arrestin-mediated epidermal growth factor receptor transactivation [8] that further up-regulates β-adrenoreceptors by several pathways [23]. Similarly, obestatin may enhance the effect of β-adrenergic signaling by a direct action on cardiac muscle cells.

Pretreatment with pertussis toxin almost entirely abolished the effect of obestatin. There is a small difference where at a concentration of 1 nmol/l the obestatin effect is statistically insignificant when compared to the effect of the same obestatin concentration in the presence of pertussis toxin. However, this difference might be explained by a direct increase of cardiac

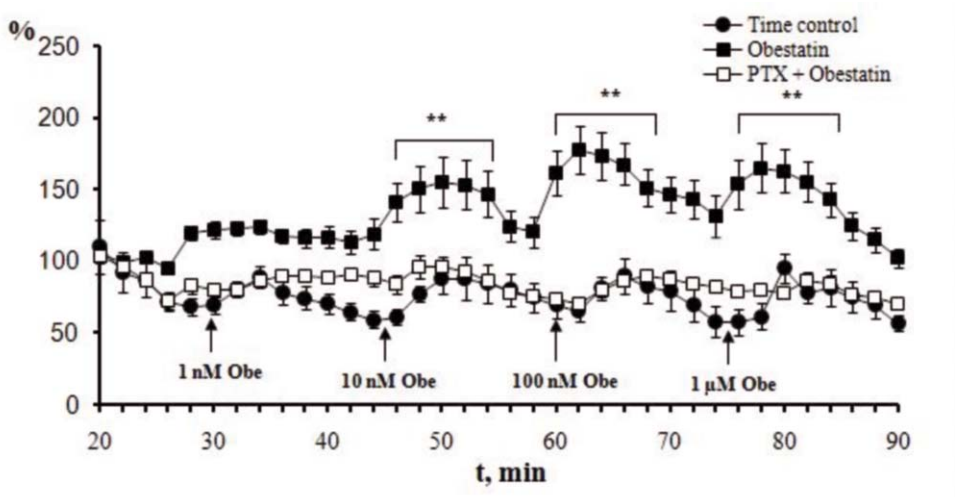

Figure 4. Effect of obestatin on the maximal force of contractions of PTX-treated frog heart. Time course, conditions of the experiments and obestatin concentrations are the same as in Figure 1. PTX (2 ng/ml) was injected 30 min before administration obestatin (□). Time control of maximal force of contractions are illustrated with (●) and obestatin contractions – with (■). Data are means ± SEM of 6 experiments. ** $P < 0.01$ *vs.* obestatin + PTX.

muscle cell excitability by G_i-protein inhibition due to removal of G_i-protein-dependent K^+ channels activation. Therefore, it is suggested that obestatin could increase the release of catecholamine neurotransmitter, which is epinephrine in amphibians [24], *via* a G_i-protein sensitive mechanism. G_i-protein participates similarly to human retinal pigment epithelial cells [25] and human gastric cancer cells [7] where it has been reported that obestatin initiates a G_i-dependent signaling that further activates phosphoinositide-3 kinase, protein kinase C and then Src family kinase for a stimulation of extracellular signal-regulated kinases. Consequently, obestatin may play the role of a periphery neuron modulator in frog hearts.

Ghrelin and obestatin exert opposite influences on cardiac tissue – ghrelin has a negative inotropic effect in rat papillary muscles of right ventricle [26] while this study demonstrates an increase of the force of contraction in the presence of obestatin in excised frog hearts. Ghrelin is actively synthesized and secreted by murine and human cardiomyocytes [27]. Thus, ghrelin and obestatin as peptides initiated from one precursor molecule (preproghrelin) and may have local regulatory effects independently of their blood plasma levels. However, it is still early to speculate about a certain functional relation or antagonism between ghrelin and obestatin as cardiac muscles modulators based on Soares *et al.* [26] and our studies due to the deficit of data and the very probable species differences.

It is concluded that obestatin *via* a G_i-protein-sensitive signaling increases the release of epinephrine from heart autonomic neurons and/or directly sensitizes the cardiac β-adrenergic receptors that further enhance the force of contraction of frog heart preparations *in vitro* by a mechanism that mainly involves the activation of PKA.

Acknowledgements

This investigation was supported by grants № 79/2007 and № 59/2008 (to H.G.) from the Sofia University 'St. Kliment Ohridski'.

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