Role of Phosphatidylinositol-3-kinase and Protein Kinase Cζ in the Adenylate Cyclase Signal Mechanism of Action of Relaxin in Muscle Tissues of Rats and Mollusks

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We showed that phosphatidylinositol-3-kinase and protein kinase Cζ are involved in the adenylate cyclase signal mechanism of relaxin action. A selective inhibitor of phosphatidylinositol-3-kinase wortmannin blocked the stimulatory effect of relaxin on adenylate cyclase in rat skeletal muscles and *Anodonta cygnea* smooth muscles. Antibodies against protein kinase Cζ abolished the relaxin-induced stimulation of adenylate cyclase in rat muscles, but not in mollusk muscles. Our results indicate that phosphatidylinositol-3-kinase and protein kinase Cζ play a role in the adenylate cyclase signal mechanism of relaxin action.

Key Words: *adenylate cyclase mechanism; insulin; relaxin*

The peptide hormone relaxin belongs to regulatory peptides of the insulin superfamily, which includes insulin, insulin-like growth factors, and insulin-like peptides of invertebrates. These peptides have similar structure and function, which reflects their common evolutionary origin. Relaxin serves as a pleiotropic endocrine and paracrine factor, which regulates functional activity of the reproductive and cardiovascular system, growth and differentiation of cells, and other processes [5]. The molecular mechanisms of action of relaxin are poorly understood. Previous studies suggest that they involve a variety of secondary messengers, including cyclic nucleotides and nitric oxide [4].

The study of molecular mechanisms of regulatory effect of insulin-like peptides revealed the adenylate cyclase signal mechanism (ACSM) for the action of insulin and related peptides in muscle tissues of vertebrates and invertebrates. This mechanism includes the following signal sequence: tyrosine kinase receptor→G_i-protein (βγ-dimer)→phosphatidylinositol-3-kinase (PI-3-K)→protein kinase Cζ (PKCζ)→G_S-protein→adenylate cyclase (AC) [3,7-10]. The evolutionary relationship between peptides of the insulin group suggests that the effect of relaxin is mediated by a similar mechanism. Early studies showed that this mechanism involves various signal proteins, including the tyrosine kinase receptor, G_S-protein, and AC [1,6]. Here we studied and compared ACSM for the effect of relaxin in skeletal muscles of rats and smooth muscles of *Anodonta cygnea* mollusks. ACSM for relaxin was compared with that for insulin.

PI-3-K is a key enzyme of phosphoinositide metabolism, which serves as a target for the regulatory effect of insulin and growth factors [2]. Secondary messengers regulate PKC of various classes. For example, atypical PKCζ is activated by phosphatidylinositol-3,4,5-triphosphate. Functionally related enzymes PI-3-K and PKCζ are involved in the regulation of cell growth, apoptosis, and metabolism. This work was designed to evaluate the role of these enzymes in ACSM for the action of relaxin. We studied the effect of wortmannin (selective inhibitor of PI-3-K) and specific antibodies against PKCζ on AC activation by relaxin.

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MATERIALS AND METHODS

The fraction of plasma membranes was isolated from hindlimb muscles of *Rattus norvegicus* rats (n=4-6) and leg smooth muscles of *A. cygnea* freshwater bivalve mollusks (n=25-30) [3,10]. Human relaxin-2 was presented by Dr. Christian Schwabe (Medical University, South Carolina, USA). Human insulin was obtained from Lilly Company. A selective and irreversible inhibitor of PI-3-K wortmannin (Sigma) blocks functional activity of the enzyme via a direct interaction with catalytic subunits. PKCζ was identified with specific antibodies against the synthetic peptide corresponding to the C-terminal region of rabbit PKCζ (577-592, Sigma).

AC activity was measured as described elsewhere [3,10]. The membrane fraction of rats and mollusks was incubated in the reaction mixture at 37 and 30°C, respectively, for 2.5 min. AC activity was determined by the amount of cAMP formed in the enzymatic reaction. cAMP content was measured by column chromatography using aluminum oxide. We studied *in vitro* effects of peptide hormones, wortmannin, and antibodies on AC activity. Wortmannin and antibodies were preincubated with the sample for 15 min. The hormones were used in a concentration producing maximum activation of AC [6,8]. The time of exposure was 2.5 min. Control samples contained the corresponding solvents.

The results were analyzed by using ANOVA software. Each experiment was performed in 3 repetitions. The data are expressed as $M\pm m$ of several independent experiments. Differences between control samples and samples exposed to the effect of hormonal and non hormonal agents were significant at $p<0.05$.

RESULTS

In series I we compared the activating effect of relaxin and insulin on AC in rat and mollusk muscles. Similarly to insulin, relaxin had a strong activating effect on AC (Table 1). These data indicate that both tissues are sensitive to peptides and, therefore, contain the corresponding receptors.

In series II we studied the mechanism of the activating effect of relaxin, which is based on coupling of the signal proteins in ACSM of insulin [3,9,10].We compared *in vitro* effects of selective PI-3-K inhibitor wortmannin (10^{-9}-10^{-7} M) and insulin (10^{-8} M) on relaxin-induced activation of AC in rat and mollusk muscles (Fig. 1). Wortmannin in specified concentrations completely blocked the stimulatory effect of relaxin and insulin on AC. It was interesting to evaluate whether wortmannin specifically inhibits the effect of insulin-like peptides on AC. The influence of wortmannin on activation of AC by biogenic amines isoproterenol (β-adrenoceptor agonist) and serotonin was determined in rat skeletal muscles and mollusk smooth muscles, respectively. The effect of biogenic amines is mediated by a short signal pathway, which includes serpentine receptor, G_S-protein, and AC. Wortmannin did not abolish activation of AC by biogenic amines. These data indicate that PI-3-K is involved in ACSM for the effect of relaxin (similarly to insulin).

In series III we studied the effect of specific antibodies against mammalian PKCζ on activation of AC by relaxin. Antibodies in dilutions of 1:1000-1:10 dose-dependently inhibited the activating effect of relaxin and insulin on AC in rat skeletal muscles (Fig. 2, *a*). These data indicate that PKCz is involved in ACSM for the effect of relaxin. Similar results were obtained in previous studies of ACSM for insulin [9].

Antibodies against PKCζ did not abolish activation of AC in mollusk skeletal muscles produced by relaxin and insulin (Fig. 2, *b*). Antibodies against mammalian PKCζ had no inhibitory effect in mollusks, which is probably related to the species specificity of these antibodies and/or differences in PKC isoforms in muscle tissues of mammals and mollusks. Therefore, PKC involved in ACSM for the effect of insulin-

TABLE 1. *In Vitro* Effects of Relaxin (10^{-8} M) and Insulin (10^{-8} M) on AC Activity in Rat Skeletal Muscles and Mollusk Smooth Muscles

Treatment	AC activity			
	rats		mollusks	
	$M\pm m$, pmol cAMP/mg protein/min	%	$M\pm m$, pmol cAMP/mg protein/min	%
No additives	19.7±1.7	100	10.2±1.0	100
Relaxin	39.8±2.0	202	28.4±1.3	278
Insulin	49.7±3.1	252	34.1±2.7	334

Note. 100%, enzyme activity in the control.

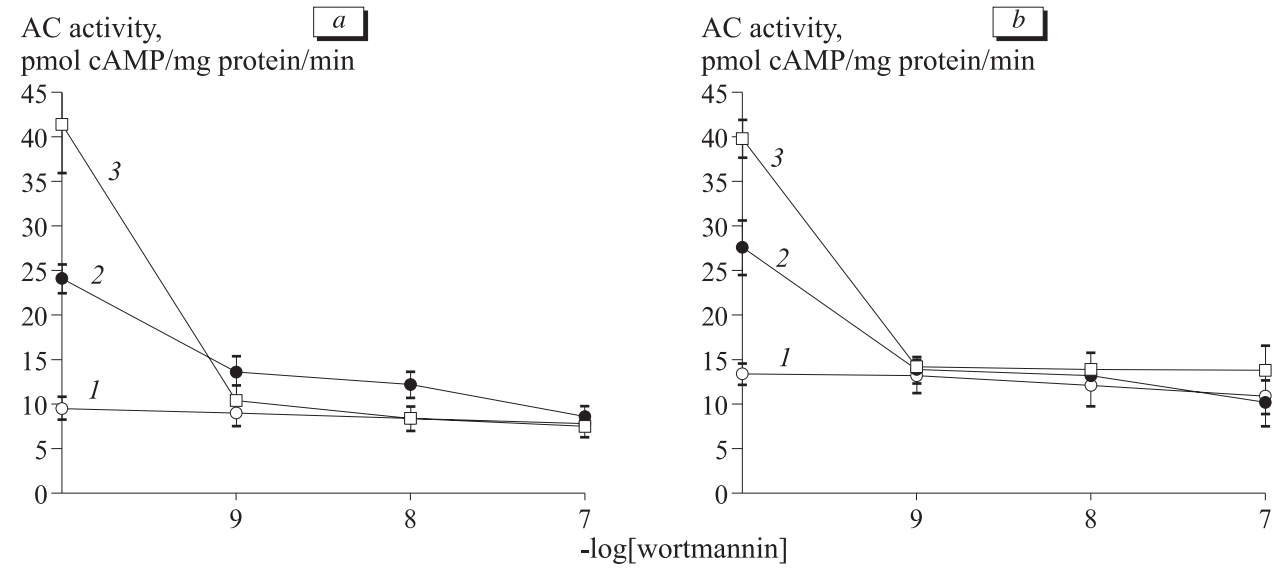

Fig. 1. Effect of wortmannin on AC activation by 10^{-8} M relaxin and 10^{-8} M insulin in rat (*a*) and mollusk muscles (*b*): control (*1*), 10^{-8} M relaxin (*2*), and 10^{-8} M insulin (*3*).

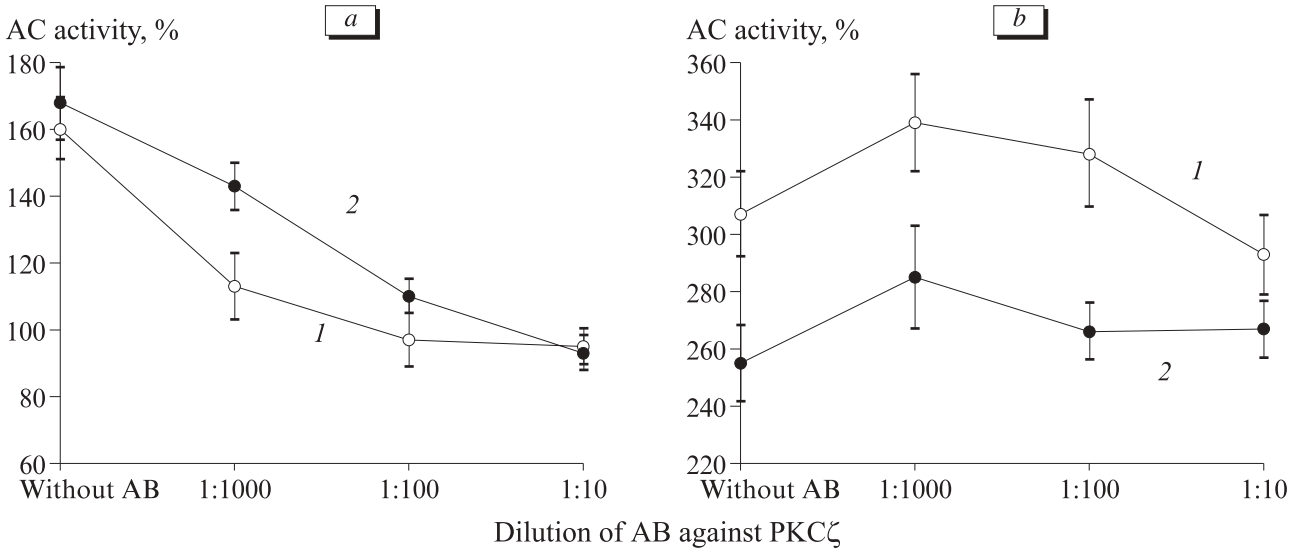

Fig. 2. *In vitro* effect of antibodies (AB) against protein kinase Cζ (PKCζ) on AC activation by 10^{-8} M relaxin (*1*) and 10^{-8} M insulin (*2*) in rat (*a*) and mollusk muscles (*b*). 100%, enzyme activity in the control.

like peptides in mollusk muscles does not belong to the group of PKCζ or differs in activity from the enzyme in vertebrates. The first assumption is confirmed by published data that calcium-independent PKC in *Aplysia californica* mollusks serves as a target for the effect of insulin realized via PI-3K and is homologous to a new isoform of mammalian PKCε [11].

Our study showed that ACSM for the effect of relaxin involves 2 signal proteins, PI-3-K and PKCζ. ACSM for the action of relaxin in rat skeletal muscles can be presented by the following signal sequence: tyrosine kinase receptor→PI-3-K—PKCζ→G_S-protein→AC. These data indicate that ACSM for the effects of relaxin and insulin have the same postreceptor stages of hormonal signal transduction. A role of inhibitory G-proteins in the mechanism of action of relaxin should be studied in further experiment.

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