ELSEVIER

Biochemical Pharmacology 61 (2001) 1277–1291

Biochemical Pharmacology

A dual role of protein kinase C in insulin signal transduction via adenylyl cyclase signaling system in muscle tissues of vertebrates and invertebrates

Svetlana A. Plesneva, Alexander O. Shpakov, Ludmila A. Kuznetsova, Marianna N. Pertseva*

Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, Thorez au. 44, St. Petersburg, 194223, Russia

Received 8 March 2000; accepted 20 September 2000

Abstract

Further decoding of a novel adenylyl cyclase signaling mechanism (ACSM) of the action of insulin and related peptides detected earlier (Pertseva *et al*. Comp Biochem Physiol B Biochem Mol Biol 1995;112:689–95 and Pertseva *et al*. Biochem Pharmacol 1996;52:1867–74) was carried out with special attention given to the role of protein kinase C (PKC) in the ACSM. It was shown for the first time that transduction of the insulin signal via the ACSM followed by adenylyl cyclase (AC, EC 4.6.1.1) activation was blocked in the muscle tissues of rat and mollusc *Anodonta cygnea* in the presence of pertussis toxin, inducing the impairment of G_i-protein function, wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3-K), and calphostin C, a blocker of PKC. The cholera toxin treatment of muscle membranes led to an increase in basal AC activity and a decrease in enzyme insulin reactivity. Phorbol ester and diacylglycerol activation of PKC (acute treatment) induced the inhibition of the insulin AC activating effect. This negative influence was also observed in the case of the AC system activated by biogenic amines. It was first concluded that the ACSM of insulin action involves the following signaling chain: receptor tyrosine kinase $\Rightarrow$ G_i ($\beta\gamma$) $\Rightarrow$ PI3-K $\Rightarrow$ PKCζ (?) $\Rightarrow$ G_s $\Rightarrow$ AC $\Rightarrow$ adenosine 3′,5′-cyclic monophosphate. It was also concluded that the PKC system has a dual role in the ACSM: (1) a regulatory role (PKC sensitive to phorbol esters) that is manifested as a negative feedback modulation of insulin signal transduction via the ACSM; (2) a transductory role, which consists in direct participation of atypical PKC (PKCζ) in the process of insulin signal transduction via the ACSM.

Keywords: Adenylyl cyclase; Insulin; Protein kinase C; Rat and mollusc muscles

1. Introduction

In the last decade, there has been an accumulation of data showing the existence in the cell of functional cross-talk between the hormone-regulated AC signaling system and PKC, which plays a key role in a number of lipid-dependent pathways of hormonal signal transduction. A modulating influence of PKC on the AC system regulated by catecholamines, vasopressin, histamine, and other hormones in different objects and tissues was shown [1–7]. The results of these investigations were very contradictory: in some cases, PKC activation by DAG and PE led to intensification of hormonal action on the AC system, in others to its weakening. Such a picture of cross-talk between the PKC and AC systems was ascribed by the authors to two circumstances: (1) The existence of different patterns of AC and PKC isoforms in various cells and tissues. Many molecular isoforms of PKC (at least 12) [8] and AC (at least 10) [9] possessing different properties have been identified. Specifically, they differ in their ability to interact with each other. The presence of the key enzyme's isoforms in cell is suggested to be of great importance in the signal processing realized in a tissue/cell-specific manner. (2) The existence of several targets for PKC action in the hormone-sensitive AC system at different levels, namely receptor, G-protein, and AC.

* Corresponding author. Tel.: +7-812-552.3117; fax: +7-812-552.3012.

E-mail address: sveta@iephb.ru (M.N. Pertseva).

Abbreviations: PKC, protein kinase C (α, λ, ε, φ, ζ, its isoforms); AC, adenylyl cyclase (EC 4.6.1.1); AC I, II, etc. (isoforms of AC); ACSM, adenylyl cyclase signaling mechanism; DAG, diacylglycerol; PE, phorbol esters; PMA, phorbol 12-myristate 13-acetate; PI3-K, phosphatidylinositol 3-kinase; G_s-protein, stimulatory GTP-binding protein; G_i-protein, inhibitory GTP-binding protein; α, β, γ, the subunits of G-proteins; IGF-I, insulin-like growth factor-I; PT, pertussis toxin; ChT, cholera toxin; Gpp[NH]p, 5′-guanylylimidodiphosphate; DTT, dithiothreitol; and cAMP, adenosine 3′,5′-cyclic monophosphate.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00592-5

Hence, the problem of functional cross-talk between these two signaling systems is very complicated. Some aspects of the problem, such as the points of interaction and the mechanisms of its realization, remain unclear. The available data in the literature are concerned with hormones acting via receptors penetrating the membrane seven times (serpentine receptors) and coupled with the AC system [1–7]. It is commonly accepted that the AC system is not involved in the mechanisms of action of hormones possessing receptors with intrinsic tyrosine kinase activity. However, our recent discovery of the participation of the ACSM in the regulatory action of insulin superfamily peptides (insulin, IGF-I, relaxin, and others) via signaling chain: receptor tyrosine kinase $\Rightarrow$ G_s-protein $\Rightarrow$ AC [10–12] has changed the situation.

The present work is devoted to the further deciphering of this ACSM and the role of PKC system in it, in particular. Proceeding from data in the literature demonstrating the activating effect of insulin on PKC [13,14] on the one hand, and the ability of PKC to phosphorylate the main components of the hormone-regulated AC system (receptor, G-protein, AC) [6,15] on the other, an attempt was made to answer the question as to whether PKC is a necessary link in the ACSM or a regulator of the process of insulin signal transduction via the AC system.

First, the role of lipid-activated, serine/threonine-specific PKC in the realization or modulation of the AC-stimulating action of insulin in rat skeletal muscles and in smooth muscles of the freshwater bivalve mollusc *Anodonta cygnea* was studied. In these muscles, the ACSM of insulin action *in vitro* and *in vivo* was revealed for the first time [10–12, 16]. The activation of PKC by insulin is realized, as was established earlier, not along the classic phosphoinositide pathway, but through the hydrolysis of phosphatidylinositol glycans and phosphatidylcholines, leading to the formation of DAG and activation of DAG- and PE-sensitive isoforms of PKC in a number of target tissues [11,13,17,18]. Atypical DAG- and PE-insensitive isoforms of PKC, specifically PKCζ regulated by insulin, are activated by lipid products of the PI3-K reaction triggered by this hormone [19–21].

To account for the role of phorbol-sensitive PKCs, the dependence of the AC-stimulating effect of hormone on their activation by DAG and its lipid-soluble analog, PMA (acute treatment imitating the insulin effect), was studied. The targets of PKC action in the insulin-regulated AC system were determined by studying the effect of the activators on different links of this signaling system, at the level of: (1) AC catalytic activity; (2) functional coupling of AC and G_s-protein as estimated by the stimulating effect on the enzyme of the non-hydrolizable GTP analog, Gpp[NH]p; and (3) receptor tyrosine kinase and the initial postreceptor links as estimated by the functional state of the insulin-activated AC signaling system as a whole.

To answer the question as to whether the influence of activated PKC on the AC system is hormone- and receptor-specific, the effect of PKC activators on the AC system regulated by biogenic amines (serotonin in the case of mollusc and isoproterenol in the case of rat) via receptor of the serpentine type was also studied. Besides transduction of the stimulatory hormonal signal, transmission via the ACSM of the inhibitory signal under the condition of PKC activation was investigated.

We next studied the role of atypical molecular isoforms of PKC in the insulin-stimulated AC system using wortmannin, a specific inhibitor of PI3-K. The latter, when stimulated by insulin, generates phosphatidylinositol-3,4,5-triphosphate, which in turn activates PKCζ. Thus far, it is not clear whether PKCζ participates in the regulation of insulin signaling or if it is directly engaged in one of the insulin-inducible signaling pathways, likely the ACSM.

As is known, PI3-K is activated by $\beta\gamma$-subunits of heterotrimeric G-proteins (with G_i their main donor) [22] that are released as a result of G-protein activation by different hormones [23]. Proceeding from this information, we attempted to show an involvement of G_i-protein in insulin-stimulated AC signaling using PT (as a tool for G_i-protein identification), which induces specific ADP-ribosylation of α_i-subunits resulting in G_i-protein inactivation [24]. In addition, the influence of ChT treatment on the insulin-stimulated AC system was investigated to reveal the involvement of G_s-protein in ACSM as suggested by us previously [10,11]. Finally, we studied the effect of calphostin C, a highly selective inhibitor of different isoforms of PKC [25], on transduction of the insulin signal via the ACSM.

Unlike many investigations devoted to this problem, our study was carried out not on whole cells, but on plasma membrane fractions isolated from rat and mollusc tissues. Membrane fraction was chosen due to the following considerations: first, the main components of the hormone-sensitive AC system (receptor, G-proteins, AC) are connected with the plasma membrane; second, according to data in the literature [15,26–29], in membrane fractions of different cells and tissues (including muscles), a number of PKC isoforms sensitive to insulin are present in the basal (non-stimulated) state, specifically PKC α, λ, ε, and φ, and atypical PKCζ. Thus, both partners (AC and PKC isoforms) whose interaction was under study are present in the same cell fraction. Third, the use of membrane fraction as compared to a whole cell has some advantages. It allowed us to study the cross-talk between AC- and PKC-dependent signaling systems at the level of cell membrane, thereby avoiding more complex intracellular processes, specifically PKC translocation. This will render interpretation of the obtained data easier. Such an approach finds confirmation in the studies of some authors [15,27] who detected interaction between AC and PKC systems in the membrane fractions of some tissues and cells.

2. Materials and methods

2.1. Chemicals and radiochemicals

Creatine phosphate, creatine phosphokinase, GTP, Gpp[NH]p, Tris–HCl, alumina for column chromatography,

ATP, cAMP, NAD, NADP, thymidine, DTT, EDTA, and imidazole were obtained from Sigma Chemical Co. [α-^{32}P]ATP (4 Ci/mmol) was from Isotope.

2.2. Hormones

Mammalian insulin (24 IU) was obtained from Eli Lilly and Co. and human recombinant IGF-I from Amersham. Isoproterenol and serotonin were obtained from Sigma.

2.3. Activators and inhibitors

DAG is a natural and PMA (both from Sigma) a synthetic activator of PKC isoforms belonging to the classical PKC and novel PKC, but they have no effect on phorbol-insensitive atypical PKC. The activating effect of DAG and PMA is likely to be realized via a common mechanism responsible for a highly selective increase in PKC affinity to phosphatidylserine-containing membranes [30].

Calphostin C (Sigma) is a perylenequinone isolated from *Cladosporium cladosporioides*. It is a potent selective inhibitor of PKC showing at least 1000-fold greater potency against this enzyme than that obtained against a range of other protein kinases [25]. The mechanism of PKC inhibition by calphostin C is suggested to involve photoactivation of the drug, resulting in the formation of a short-lived species that interacts with the cysteine-rich zinc-finger structure of the PKC regulatory domain. This results in irreversible inactivation of PKC.

Wortmannin (Sigma) isolated from *Penicillium wortmannii* is a fungal metabolite that acts as a potent selective and irreversible inhibitor of PI3-K [31]. The mechanism of its action consists in blocking the activity of PI3-K as a result of direct interaction with its catalytic subunit. Wortmannin also inhibits other kinases, such as myosine light chain kinase and PI4-K, but at a concentration 100-fold higher than that required for inhibition of PI3-K.

The effects of insulin and the activators and blockers mentioned above were studied *in vitro*. The agents were added to the samples for determination of AC activity. Insulin was dissolved in 0.01 N HCl, serotonin and isoproterenol were dissolved in Tris–HCl (pH 7.5), and DAG, PMA, and calphostin C in DMSO. Stock solutions of these agents were diluted with Tris–HCl (pH 7.5) to necessary concentrations immediately before use. The activators and blockers were preincubated with the samples for 10 min (DAG, PMA, calphostin C) and for 5 min (wortmannin) followed by addition of insulin or other hormones (serotonin, isoproterenol) at the concentration giving a maximal AC-stimulating effect [10]. The duration of action of insulin was limited to 2.5 min, a time sufficient for the AC effect to reach its peak [10]. In the case of serotonin or isoproterenol, the duration of action was limited to 10 min. Samples treated in the same manner with the medium used to dissolve the hormones, activators, and blockers served as control.

2.4. Bacterial toxins

PT (Sigma), an exotoxin isolated from the culture of *Bordetella pertussis*, has proved to be an invaluable tool in the characterization of G_i- and G_o-proteins associated with different signaling systems. The toxin catalyzes the ADP-ribosylation of $G_{i\alpha}$ (cysteine residue), leading to its inactivation and blockade of hormonal inhibition of AC activity [24,32].

ChT A subunit (Sigma) was isolated from *Vibrio cholerae*. This agent induces the ADP-ribosylation of arginine residue in $G_{s\alpha}$. Such modification inactivates the GTPase and maintains $G_{s\alpha}$ in a permanently active state. As a result, cholera toxin treatment enhances the catalytic activity of AC and decreases its response to the regulatory effects of hormones acting via G_s [24,32].

2.5. Membrane preparation

Sarcolemma membrane fractions were isolated from the foot (smooth) muscle of the freshwater bivalve mollusc *Anodonta cygnea* and the leg skeletal muscle of the rat *Rattus norvegicus* (for each fraction 25–30 molluscs and 4–6 rats were used) according to the method of Kidwai *et al*. [33], with our modifications. Pieces of muscle tissue were homogenized in 0.25 M sucrose/40 mM Tris–HCl (pH 7.5) buffer (buffer A). Homogenate was centrifuged at $1000 \times g$ for 10 min. Supernatant was centrifuged at $100{,}000 \times g$ for 1 hr. The supernatant was then removed and the pellet resuspended in buffer A and centrifuged in sucrose density gradient (0.25 M : 0.8 M) at $100{,}000 \times g$ for 1.5 hr. Vesicles located between 0.25 and 0.8 M sucrose were collected and washed with 40 mM Tris–HCl (pH 7.5) buffer using centrifugation at $100{,}000 \times g$ for 1 hr. Pellet was resuspended in 50 mM Tris–HCl (pH 7.5) buffer and used for AC activity assay.

The membrane fractions were characterized by the activity of marker enzymes such as 5′-nucleotidase (EC 3.1.3.5) and Na,K-ATPase (EC 3.6.1.3) and by electron microscopy (data are not shown). According to these criteria, the obtained preparations are fractions enriched in external membranes of the muscle cell. As a result, 12- to 15-fold purification of AC was achieved. These data are consistent with our data described earlier [34]. The membrane preparations were very similar in terms of protein concentration (nearly 3 mg/mL) and enrichment with marker enzymes.

2.6. Adenylyl cyclase assay

AC (EC 4.6.1.1) activity was measured using the method of Salomon *et al*. [35], with some modifications. The reaction mixture (final volume 50 μL) contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM ATP, 1 μCi [α-^{32}P]ATP, 1 mM cAMP, 20 mM creatine phosphate, 0.2 mg/mL of creatine phosphokinase, and 15–20 μg of the

membrane protein. Incubation was carried out at 37° (rat) and 30° (mollusc) for 2.5 min (insulin) and 10 min (serotonin and isoproterenol). The reaction was initiated by addition of the membrane protein and terminated by adding 100 μL of 0.5 N HCl and immersing the tubes in a boiling water bath for 7 min. One hundred microliters of 1.5 M imidazole was added to each tube. Under the assay condition, the AC activity was linear. cAMP formed as a result of the enzyme reaction was separated according to White's method [36], using alumina for column chromatography. The samples were placed on neutral alumina columns and cAMP was eluted by 8 mL of 10 mM imidazole–HCl buffer (pH 7.4). The eluates were collected in scintillation vials and counted using an LKB 1209/1215 RackBeta scintillation counter. Each assay was carried out in triplicate at least three times, and the results were expressed as pmol cAMP/min per mg of membrane protein.

2.7. ADP-ribosylation procedure

The samples of membrane fraction (concentration of the membrane protein was 0.95–1.0 mg/mL) were incubated at 30° (for mollusc) or 37° (for rat) for 45 min with or without 10 μg/mL of PT or 100 μg/mL of ChT in 400 μL of 50 mM Tris–HCl (pH 7.8), 2 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, 0.1 mM NAD, 1 mM NADP, 0.1 mM GTP (for PT) or Gpp[NH]p (for ChT), 1 mM ATP, and 10 mM thymidine. The toxins were previously activated in the presence of DTT and ATP for 15 min at 37°. After ADP-ribosylation, the suspension was diluted to a final volume of 5 mL with an ice-cold 50 mM Tris–HCl (pH 7.5) buffer and centrifuged at $100{,}000 \times g$ for 30 min. Pellets were resuspended in 50 mM Tris–HCl (pH 7.5) buffer and immediately used for determination of AC activity.

2.8. Protein assay

The protein content was determined according to the method of Lowry *et al*. [37] using BSA as a standard.

2.9. Statistical analysis

All data are presented as the means ± SEM. Differences between control and the hormone-, activator-, and blocker-treated groups were statistically assessed using one-way ANOVA and considered significant at $P < 0.05$.

3. Results

3.1. The effect of DAG and PMA on basal, Gpp[NH]p-, and hormone-regulated AC activity

At the concentrations of 10^{-10}–10^{-7} M, both agents (DAG and PMA) *in vitro* stimulated the catalytic activity of AC in rat muscle membranes by 34–140% over the control and in mollusc membranes by 39–140% (Fig. 1). The effect was most pronounced in the concentration range of 10^{-9}–10^{-8} M and diminished at a larger dose (10^{-7} M). PMA stimulation of AC basal activity in rat skeletal muscle was also observed by other investigators [38]. However, activation of PKC by DAG (Fig. 2) and PMA (data not shown) in rat and mollusc muscle membranes led to complete inhibition of Gpp[NH]p-stimulated AC activity. This provides evidence for the impairment of functional interaction between G_s-protein and AC.

In the case of PMA addition (10^{-10}–10^{-7} M), the AC-stimulating effect of insulin (*in vitro*, 10^{-8} M, 2.5 min) signal transduction via the AC system is decreased in rat (Fig. 3) (from 100% to 46–54%) and in mollusc (Fig. 3) (from 100% to 38–19%) muscles. An analogous phenomenon was observed in the mollusc and rat muscles in the presence of DAG (data not given).

To establish the specificity of the effect of PKC activation induced by PMA and DAG on insulin signal transduction, the AC-stimulating effect of the other hormones mediated via serpentine receptors (serotonin for mollusc, isoproterenol for rat) was studied under the same conditions. As is shown in Fig. 4, in the presence of DAG and PMA the effect of biogenic amines vanished in mollusc and rat. Consequently, PKC inhibition of stimulatory signal transduction via the AC system is not hormone- or receptor-specific.

In the following series of experiments, the influence of PKC activators on inhibitory signal transmission was investigated. The AC inhibitory effect of isoproterenol in the muscle tissue of the mollusc *Anodonta cygnea* was taken as a model. There, we found that unlike the vertebrate tissues, β-adrenergic agonist (isoproterenol) induces inhibition of AC activity through the β_2-adrenergic receptor and G_i-protein (G_{i2}) [39]. As is shown in Fig. 5, the AC inhibitory effect of isoproterenol in the mollusc muscle membranes (white bars without and with isoproterenol) is virtually the same in the case of DAG and PMA activation of PKC. This result is in good agreement with the data of Morimoto and Koshland [3], which suggests that the interaction between the PKC- and AC-dependent signaling systems takes place only in the case of transduction of a stimulatory signal via G_s-AC. It was concluded that the cross-talk between these signaling systems occurs only when they both are in the activated state.

3.2. The effect of wortmannin, bacterial toxins, and calphostin C on insulin-signal transduction via the AC system

Wortmannin *in vitro* (10^{-8} M) slightly reduced basal AC activity in mollusc muscle membranes (Fig. 6). In the presence of wortmannin, the AC-stimulating effect of insulin was not found. A similar picture was observed in rat muscles (data not shown).

PT treatment of the muscle membranes of mollusc and

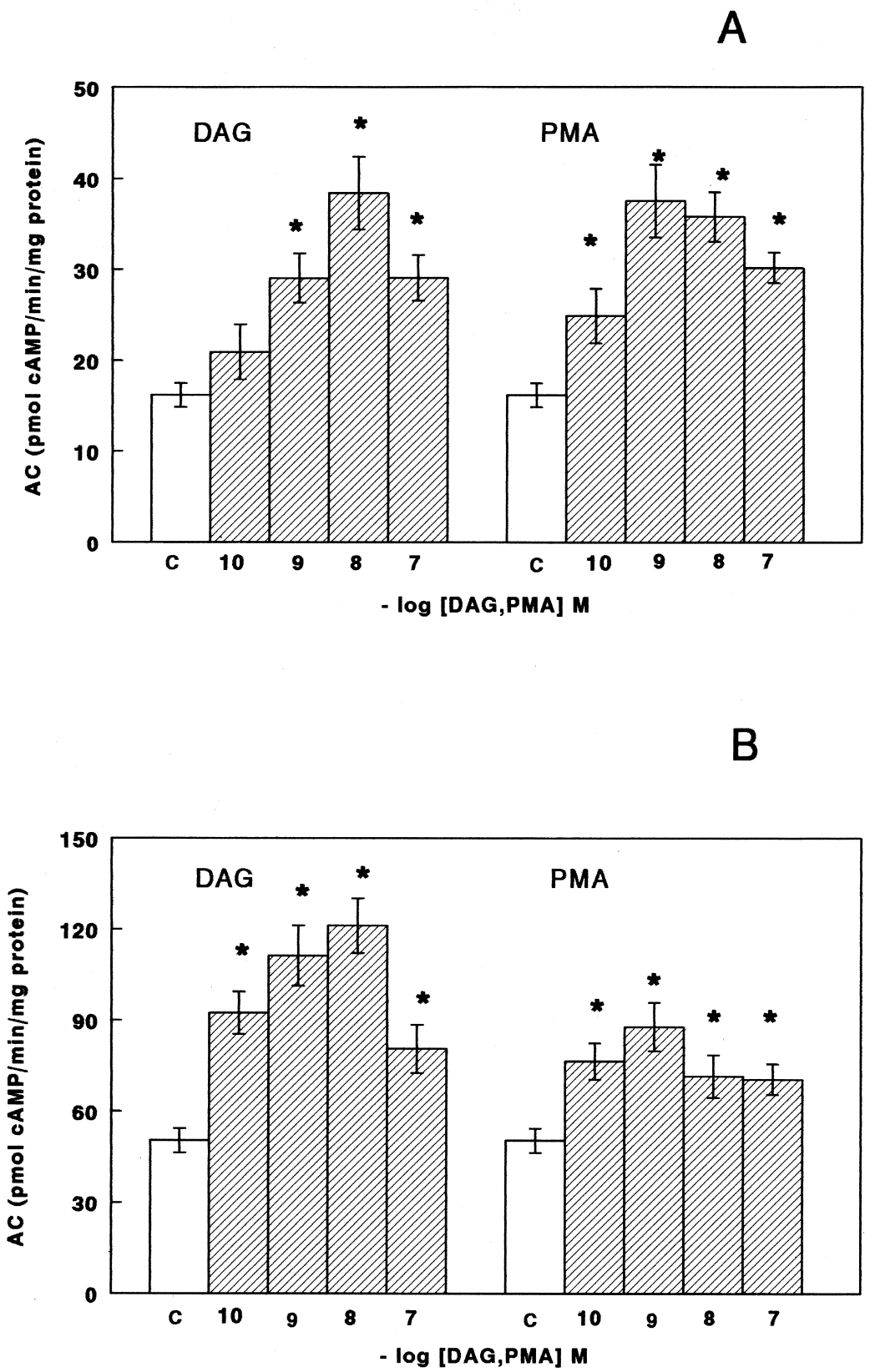

Fig. 1. Influence of DAG (10^{-10}–10^{-7} M) and PMA (10^{-10}–10^{-7} M) on basal AC activity in muscle membranes of (A) rat and (B) mollusc. Vertical axis (here and in the other figures): AC activity in pmol cAMP/min/mg of protein; Horizontal axis: (−) log of DAG or PMA concentration (M). C: control (white bars) without DAG and PMA. Statistical significance of differences from the controls is indicated by $*P < 0.05$. Here and in the other figures, each point represents the mean ± SEM for 4 values. The plotted data are from a typical experiment. N = 3–4.

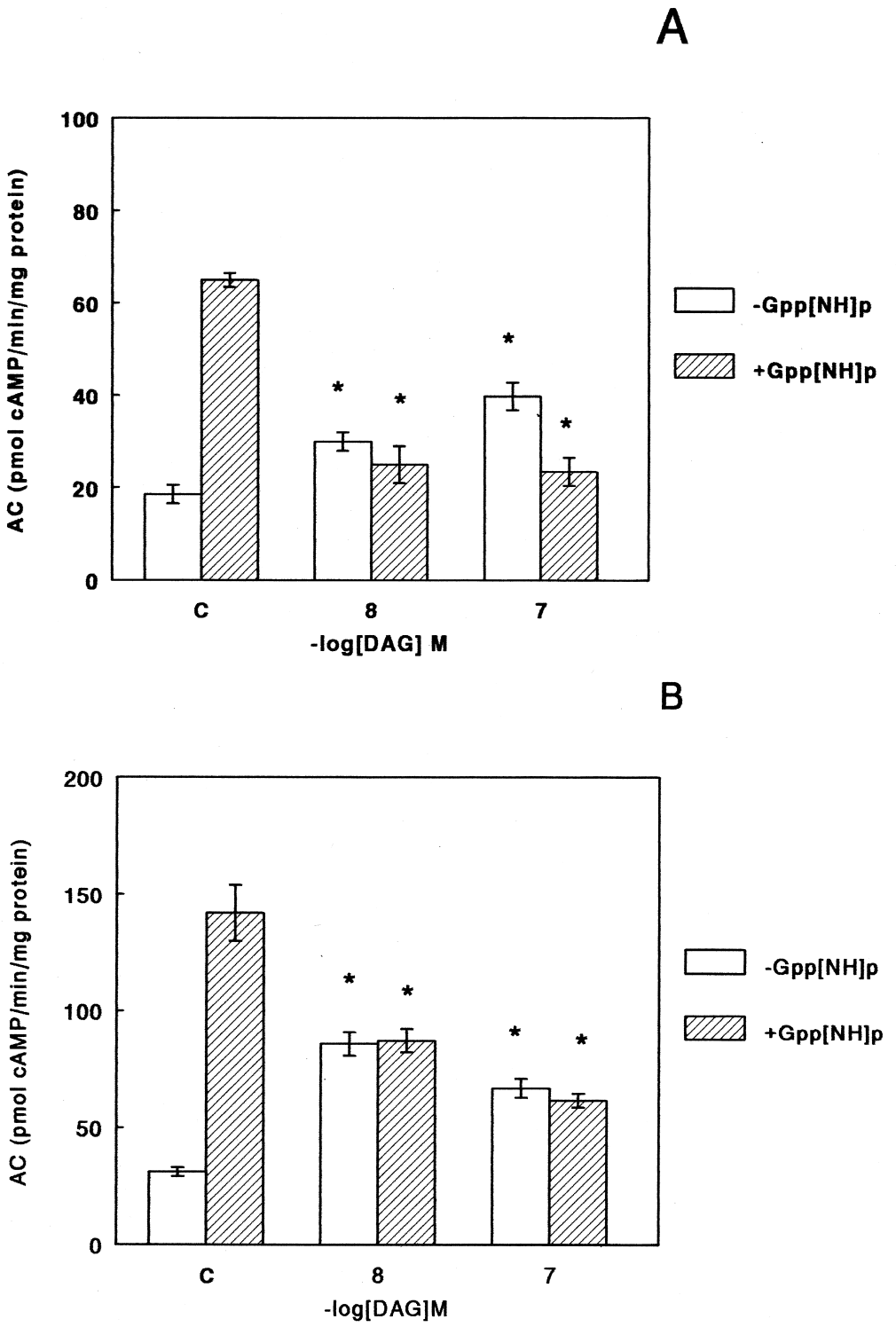

Fig. 2. Influence of DAG (10^{-8}–10^{-7} M) on Gpp[NH]p-stimulated AC activity in the muscle membranes of (A) rat and (B) mollusc. Vertical axis as in Fig. 1. Horizontal axis: (−) log of DAG concentration (M). C: control group without DAG. Concentration of Gpp[NH]p used was 10^{-6} M. Statistical significance of differences between white and hatched bars in the control and DAG treatment groups is indicated by $^{*}P < 0.05$.

rat (Table 1) led to a slight increase in AC basal activity (+9–22%) and to a significant decrease in the AC-stimulating effect of insulin (10^{-9} M) (from 282% to 8% in mollusc and 71% to 2% in rat) in the absence and presence of PT, respectively. Analogous data were obtained in the case of the AC-stimulating effect of IGF-I (Table 1). According to the mechanism of PT action [24,32], its effect is induced via ADP-ribosylation of G_i-protein and is followed

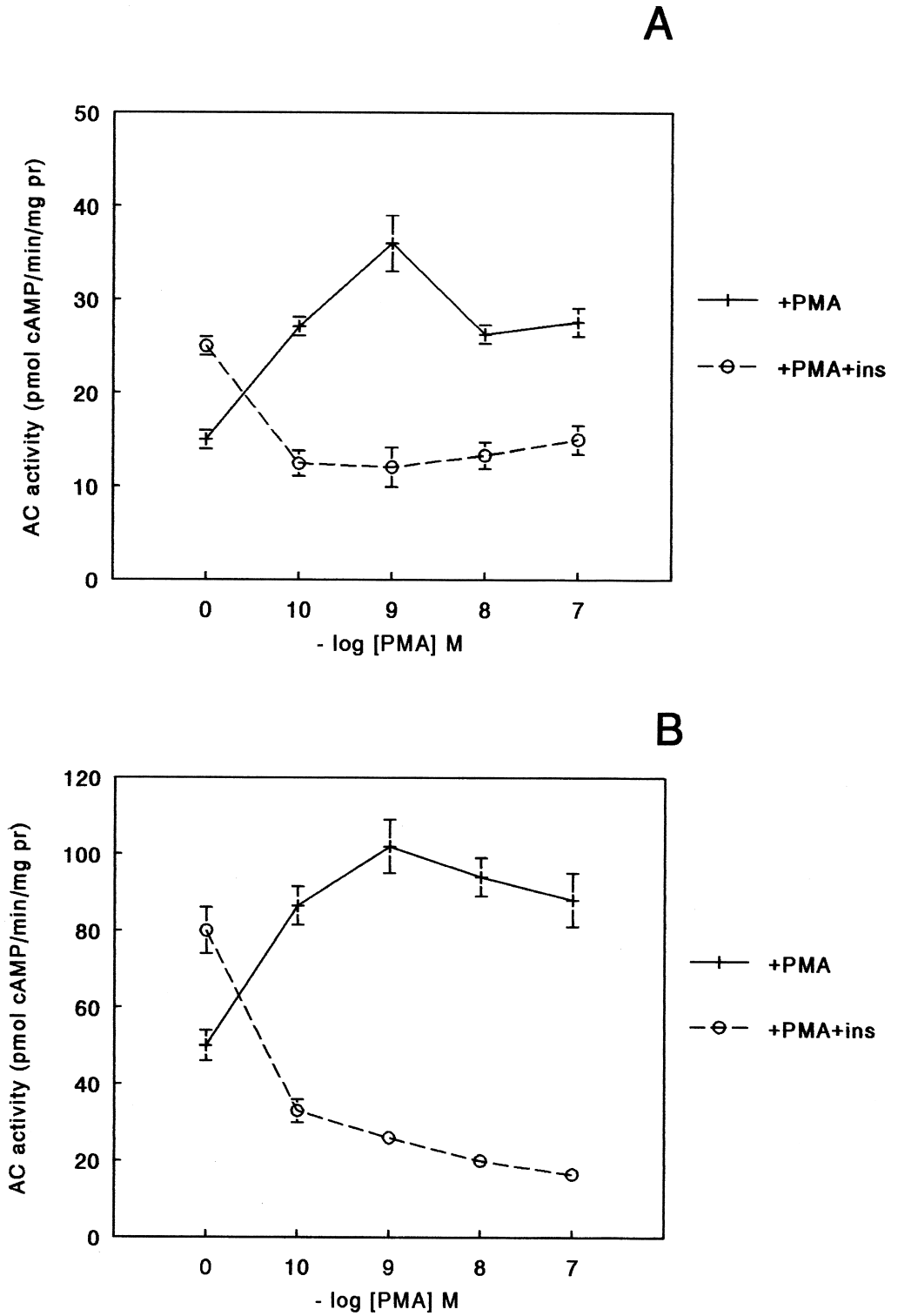

Fig. 3. Effect of PMA (10^{-10}–10^{-7} M) on insulin-stimulated AC activity in the muscle membranes of (A) rat and (B) mollusc. Vertical axis as in Fig 1. Horizontal axis: (−) log of PMA concentration (M). Concentration of insulin used was 10^{-8} M. A statistical significance of differences for each curve from the control was found ($P < 0.05$).

by loss of its functional activity. As a result, the G_i-protein inhibitory influence on basal AC activity is abolished, and its function in hormone signal transduction is impaired. However, the latter effect of PT is usually associated with transduction of inhibitory signals. In our case, PT treatment led quite unexpectedly to the blockade of stimulatory signal transmission, likely connected with the loss of ability of G_i-protein subunits to dissociation and release of $\beta\gamma$-dimer.

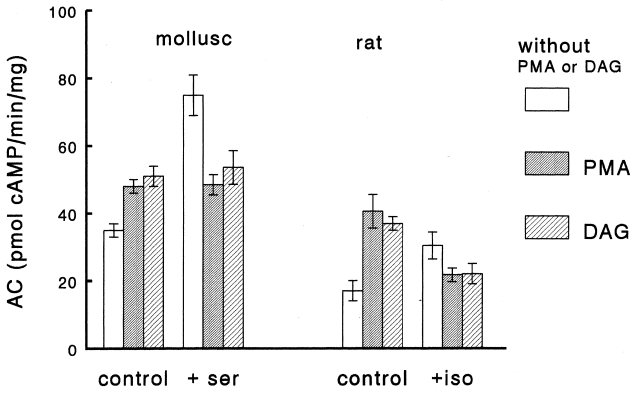

Fig. 4. Effect of PMA (10^{-8} M) and DAG (10^{-8} M) on serotonin and isoproterenol AC-stimulated activity in the muscle membranes of mollusc and rat. Vertical axis as in Fig 1. Horizontal axis: effect of PMA and DAG in the absence (control) and presence of serotonin (10^{-5} M) (for mollusc) or isoproterenol (10^{-5} M) (for rat).

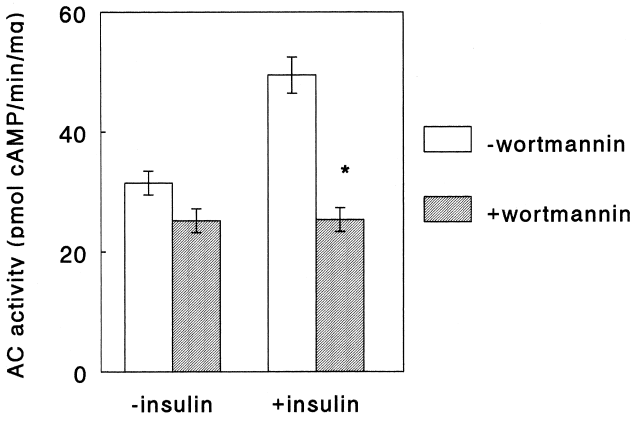

Fig. 6. Effect of wortmannin (10^{-8} M) on insulin-stimulated AC activity in mollusc muscle membranes. Vertical axis as in Fig 1. Horizontal axis: effect of wortmannin in the absence (control) and presence of insulin (10^{-5} M). Statistical significance of differences between white and hatched bars in the group with insulin is indicated by $*P < 0.05$.

As is known, $\beta\gamma$-dimer mediates a number of stimulatory signals, e.g. the activating effect on PI3-K [9,21].

ChT treatment of mollusc and rat muscle membranes led to an increase in AC basal activity (+99% and +101%, respectively; Table 1), which is ascribed [24,32] to stabilization of the GTP-binding form of $G_{s\alpha}$ subunit and to inhibition of its GTPase activity, both leading to the permanently activated state of G_s-protein. Being highly activated, AC then loses reactivity to the hormone. Indeed, in our experiments with ChT (Table 1), the insulin- and IGF–I–AC–stimulating effect was weaker compared to the control (without toxin). It should be pointed out that the combined effect of ChT and insulin on AC was less than additive (Table 1). This implies that the target for ChT and insulin action was the same G-protein, namely the G_s-protein, which supports our idea about the involvement of this G-protein in the ACSM of insulin action.

Calphostin C *in vitro* (10^{-11}–10^{-7} M) (Table 2) increased AC basal activity in the muscle membranes of mollusc and rat, but decreased insulin-stimulated AC activity in a dose-dependent manner. The most pronounced effect was observed at the concentration of 10^{-8} M. That calphostin C indeed inhibits the insulin AC effect is confirmed by the negative values of the calphostin C effect (Table 2) in a number of experiments (six out of ten). If calphostin as an inhibitor did not block the insulin AC effect, then the stimulating effect of this agent and insulin must be additive, i.e. the total effect will be positive. Calphostin C also had an inhibitory action on Gpp[NH]p- and insulin+Gpp[NH]p-stimulated AC activity (data not shown). Thus, the blockers of PI3-K, G_i-protein, and PKC used in our study exercised an inhibitory influence on insulin signal transduction via the ACSM.

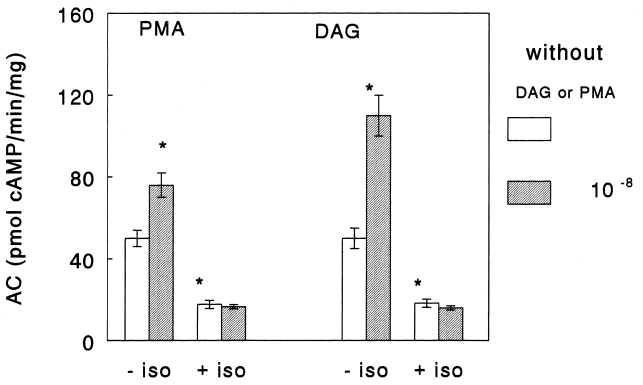

Fig. 5. Effect of PMA and DAG (10^{-8} M) on isoproterenol-inhibited AC activity in mollusc muscle membranes. Vertical axis as in Fig 1. Horizontal axis: effect of PMA and DAG in the absence (control) and presence of isoproterenol (10^{-5} M). $*P < 0.05$.

4. Discussion

To begin with, there is a great similarity in the data obtained in all experiments on the muscle tissues of the representatives of mammals (rat) and molluscs (*Anodonta cygnea*), which renders the discussion easier. This is in good agreement with the statement concerning the common principles of the structural-functional organization of AC- and PKC-dependent signaling systems in vertebrates and invertebrates [40,41].

It follows from the facts obtained in this work that the DAG- and PMA-activated PKC system has several targets of action in the insulin-regulated AC system. The first is at the level of AC, whose catalytic activity was appreciably intensified under the condition of PKC activation by DAG and PMA. According to data in the literature, PMA can either increase the catalytic activity of AC or decrease it. The direction of the effect depends on the type of isoforms of PKC and AC in the tissue under study. An increase in the AC catalytic activity (AC isoforms I, II, and III) in response

Table 1
Effect of bacterial toxin treatment of muscle membranes of mollusc and rat on basal, insulin-, and IGF-I stimulated AC activity

	AC activity (pmol cAMP/min/mg of protein)				
	Mollusc			Rat	
	−PT	+PT		−PT	+PT
Basal	63.2 ± 4.1	69.1 ± 9.6 [+ 9%]	Basal	39.7 ± 3.4	48.5 ± 2.0 [+ 22%]
Insulin 10^{-9} M	241.5 ± 18.1 (+ 282%)	74.2 ± 7.6 [+ 8%]	Insulin 10^{-9} M	67.9 ± 3.6 (+ 71%)	48.2 ± 2.7 [+ 2%]
IGF1 10^{-9} M	139.2 ± 12.4 (+ 120%)	76.8 ± 7.3 [+ 11%]	IGF1 10^{-9} M	57.4 ± 2.1 (+ 45%)	43.1 ± 1.6 [− 11%]
	−ChT	+ChT		−ChT	+ChT
Basal	17.4 ± 1.0	34.6 ± 3.6 [+ 99%]	Basal	39.6 ± 2.6	79.7 ± 2.7 [+ 101%]
Insulin 10^{-9} M	65.6 ± 4.8 (+ 276%)	48.9 ± 2.5 [+ 42%]	Insulin 10^{-9} M	69.3 ± 2.8 (+ 75%)	105.8 ± 7.4 [+ 33%]
IGF1 10^{-9} M	36.7 ± 2.4 (+ 111%)	45.0 ± 1.6 [+ 30%]	IGF1 10^{-9}M	56.7 ± 4.2 (+ 43%)	106.2 ± 6.5 [+ 33%]

Values are expressed as means ± SEM for six experiments. Figures in round brackets represent the stimulating effect of peptides in %, those in square brackets the effect of insulin and IGF-I in % in the presence of the toxins.

to the action (10 min) of PMA was observed in different cells, while AC isoforms IV, V, and VI showed weak or no response [9,42,43]. PKCα was found to be capable of increasing the catalytic function of AC isoform II [44]. There is a tendency to consider an increase in the catalytic activity of some AC isoforms as the result of elimination of the G_i-protein inhibitory effect on AC caused by the loss of α_i-subunit function due to PKC phosphorylation [9]. It has been reported that α-subunits of two G-proteins, G_i and transducin, as well as the β-subunit of the latter are all substrates of the phosphorylation of Ca^{2+}-phospholipid-dependent PKC [45].

The second target is at the level of G_s-protein and its interaction with AC as estimated by the stimulatory Gpp[NH]p effect on the enzyme. This effect was weaker after DAG and PMA treatment of the muscle membranes. The available data shows that PKCα, whose effect in cell is dependent on the type of AC isoforms, regulates their reactivity to G-proteins in different ways [44]. Interaction between PKCα and AC isoform II leads to an increase in G-protein $\beta\gamma$- and α-subunit AC-regulated activity. On the contrary, PKCα has a weak influence on the G-protein $\beta\gamma$-stimulated activity of AC isoform IV, but markedly decreases the G-protein α-subunit-stimulatory effect on the enzyme. It is via these AC isoforms that the integration of hormonal signals generated by receptors coupled with G_s-, G_i-, and G_q-proteins and mediated through their α- and/or $\beta\gamma$-subunits and PKC occurs. α-Subunits of some G-proteins ($G_{\alpha15}$ and $G_{\alpha16}$) belonging to the G_q-protein family were shown to be targets for the modifying action of PKC, which leads to their

Table 2
The AC-stimulating effect of insulin (10^{-8} M) in the presence of different concentrations (10^{-11}–10^{-7}M) of calphostin C

Calphostin concentration (M)	AC activity (pmol cAMP/min/mg of protein)			
	Mollusc		Rat	
	−insulin	+insulin	−insulin	+insulin
0	11.8 ± 1.1	41.3 ± 2.1 [+ 250%]	11.1 ± 1.3	17.8 ± 1.1 [+ 60%]
10^{-11} M	23.9 ± 1.8 (+ 102%)	37.6 ± 2.7 [+ 57%]	23.4 ± 1.1 (+ 111%)	15.7 ± 1.4 [− 33%]
10^{-10} M	25.3 ± 2.2 (+ 114%)	34.3 ± 2.6 [+ 36%]	25.7 ± 1.2 (+ 132%)	16.6 ± 1.4 [− 35%]
10^{-9} M	29.6 ± 1.7 (+ 151%)	37.5 ± 1.6 [+ 27%]	24.3 ± 2.4 (+ 119%)	14.6 ± 1.0 [− 40%]
10^{-8} M	37.7 ± 1.3 (+ 220%)	33.4 ± 1.7 [− 11%]	31.1 ± 2.4 (+ 180%)	17.5 ± 1.7 [− 44%]
10^{-7} M	28.9 ± 2.5 (+ 145%)	29.3 ± 1.5 [+ 2%]	22.1 ± 2.0 (+ 99%)	14.8 ± 1.0 [− 33%]

Values are expressed as means ± SEM for four experiments. Figures in round brackets represent the stimulating effect of calphostin C in %, those in square brackets the effect of insulin in the presence and absence of calphostin C.

phosphorylation [46]. PKC serine/threonine phosphorylation of G-proteins occurs in the $\beta\gamma$-binding region of its α-subunit. This process results in inhibition of hormone-induced signaling by the prevention of G-protein trimer association.

The third target of activated PKC such as insulin receptor tyrosine kinase could not be excluded either. PMA-activated PKC induces a reduction in the tyrosine kinase activity of insulin receptor that weakens the tyrosine phosphorylation of the receptor protein and its substrates [47,48]. In a study carried out on the culture of CHO (Chinese hamster ovary) cells with over-expressed PKCα [48], activation of the latter led to the inhibition of signal transduction induced by insulin and other insulin superfamily peptides (e.g. IGF-I). This antagonism manifests itself on the stage of ligand-stimulated tyrosine phosphorylation of several endogenic substrates of insulin receptor such as IRS 1–4 (insulin receptor substrate), PI3-K, GAP (GTPase-activating protein), and Shc (protein related to endogenic substrate for insulin receptor). It follows that in our study the blockade of insulin signal transduction via the AC system in the case of PKC activation most likely occurs at the level of G_s-protein and hormonal receptor. The functions of these signaling molecules are suggested to be impaired due to their serine/threonine phosphorylation [45–47].

According to the data presented here and the results obtained by other authors [4,27,49], inhibition of stimulatory signal transmission also takes place in the AC system regulated by hormones acting via serpentine receptors. It was suggested that in these signaling systems there are several points of PKC negative influence such as receptor, G_s-protein, AC, and the interaction between them. The negative effect is likely to involve G_i-protein, since it is eliminated as a result of PT treatment of the cells. Thus, insulin and other hormones activating PMA-sensitive isoforms of PKC induce (according to the principle of feedback regulation) the process of phosphorylation of the molecular components of the hormone-sensitive AC system, which results in a blockade of stimulatory signal transduction. The cross-talk for both partners, PKC and AC, is isoenzyme-specific [9]. Below, isoenzyme specificity will be discussed from the point of view of investigations on mammalian skeletal muscles, because there are few data on PKC and AC isoenzyme spectra in mollusc tissues.

In rat skeletal muscles, four isoforms of PE-sensitive PKC have been identified, i.e. PKCα, PKCβ, PKCε, and PKCφ; these are present both in the basal and hormone-stimulated state in membrane and cytosolic fractions [28]. The insulin-induced formation of DAG and the activation of these PKC isoforms were shown in rat skeletal muscles [28,50]. The fact that the insulin effect on PKC took a short time (1–5 min) to be felt *in vitro* is probably due to the direct interaction of PKC with insulin receptor and the tyrosine phosphorylation of the enzyme [28,29,38,51,52]. In the nervous tissue of marine mollusc *Aplysia californica*, two insulin-activated forms of PKC have been identified: Ca^{2+}-activated PKC (Apl I), an analog of conventional PKC isoforms (α, β, and γ); and Ca^{2+}-independent PKC (Apl II), similar to the novel PKC (δ, ε, η, and φ isoforms) of vertebrates [41,53].

As far as the isoenzyme spectrum of AC in the skeletal muscles of rodents (mouse) is concerned, the predominant AC isoforms are II (53%) and VI (16%), whereas AC isoform V is present only in low quantity [54]. In the peripheral tissues, AC isoforms II and V are regulated by PMA-sensitive PKC (specifically PKCα) [55]. Here, our attention was focused on the insulin-regulated AC isoforms. After our discovery of the novel ACSM of insulin action, other authors showed that the target of the stimulating effect of this hormone is AC isoform V, which can be phosphorylated with PE-sensitive PKCα and PE-insensitive PKCζ [6,55,56]. In addition, AC isoform II, which serves as integrator of different hormonal and growth factor signals passing through the heterotrimeric G-proteins and PKC system, cannot be excluded either [57]. It follows that cross-talk between the insulin-regulated PKC and AC systems the muscle tissues may involve (on the PKC side) PE-sensitive PKCα, PKCβ, and PKCφ isoforms, and (on the AC side) AC isoform V and possibly AC isoform II. Taking into account the well-known facts concerning the ability of all AC isoforms to be activated with G_s-protein [9], it is possible that some other molecular variants of the enzyme are also targets for the insulin-stimulating effect, mediated, as we suggest, via G_s-protein.

Some results of our study of wortmannin and bacterial toxin effects on the insulin-stimulated AC system are worthy of discussion. Wortmannin, being a selective inhibitor of PI3-K, blocks transduction of the insulin signal via the AC system in rat and mollusc muscle tissues. At the same time as slightly inhibiting AC basal activity, this agent distinctly suppresses insulin-stimulated AC activity. According to data in the literature [31,58], wortmannin directly interacts with the 110-kDa catalytic subunit of PI3-K, thereby preventing production of lipid-signaling molecules and thereby weakening the insulin activation of atypical PKCζ. It was shown that other insulin-signaling pathways leading to the metabolic (glucose transport) and mitogenic effects of the hormone are also blocked [14,59]. Weakening of insulin activation of Ca^{2+}-independent PKC (isoform Apl II) by wortmannin was observed in the nervous tissue of the mollusc *Aplysia californica* [53].

In discussing our data on wortmannin inhibition of the AC insulin effect in muscle membrane fraction, it should be taken into account, that PI3-K, in the non-stimulated state, is localized for the most part within the cell [19,20]. However, data in the literature suggest the existence of PI3-K isoforms that are initially connected with the structural elements of plasma membrane, in particular with cytoskeleton underlying the membrane and therefore co-purifying with it [60]. In our experiments, the inhibitory effect of wortmannin detected in the membrane fractions confirms such a suggestion. This is in good agreement with observa-

tions showing a close functional connection of PI3-K and its products with plasma membrane [60].

The inhibition of insulin signal transduction via the AC system as a result of PT treatment of muscle membranes found in the present study implies the involvement of G_i-protein in this process. Published data show that receptors coupled with G_i-protein can exercise a stimulating action on effector proteins due to liberation of G_i-protein $\beta\gamma$-subunits [9,61]. This pathway was shown for PI3-K and other downstream stages of mitogenic signaling. Moreover, activation of the receptor/G_i-protein system (α_2-adrenoreceptor) was shown to lead to potentiation of G_s-stimulated AC activity, specifically AC isoform II [62]. These data support our suggestion concerning the participation of G_s-protein in the AC signaling mechanism of action of insulin and other peptides of the insulin superfamily [10–12]. The classic effect of ChT on the G_s-AC system, which we observed in our experiments (Table 1), speaks in favor of such suggestion. The surprising discovery of inhibition by PT of the insulin stimulatory effect on AC observed in the present study and our interpretation of this fact find conformation in the work of Marjamaki *et al*. [63]. Investigating the integration of different signals directed to AC (AC II, IV, and other enzyme isoforms) revealed that ligand activation of α_2-adrenoreceptor induces both positive ($G_{\beta\gamma}$) and negative ($G_{i\alpha}$) signals that act on $G_{s\alpha}$-stimulated AC and that both signals are blocked by PT.

The facts obtained in the present work suggest the involvement of the following additional components in the ACSM: G_i-protein, PI3-K, and PKCζ. This finds support in studies showing that among PKC isoforms insensitive to PE, the target for the activating action of insulin and other growth factors is PKCζ, whose activation occurs via the PI3-K-signaling pathway [14,17,56]. It deserves mention at this point that in the case of combined expression in the cell of PKCζ and AC isoform V, the stimulating effect of insulin on the process of cAMP formation is potentiated [6].

The results of experiments with calphostin C also support this view. Calphostin C is likely to be an inhibitor of all groups of PKC known so far (both PE-sensitive and atypical isoforms) [25]. Indeed, it has been shown that in human dermal fibroblasts PKCζ is sensitive to the blocking action of calphostin C [64]. In our experiments, calphostin C interrupted insulin signal transduction through the AC system (Table 2). Since this agent increased the basal AC activity, the target of its inhibitory influence, i.e. PKC, must be located upstream in the insulin-regulated AC signaling system.

As for PKC isoforms that can be directly involved in AC-mediated insulin signaling, the group of PE-sensitive PKC is to be excluded. We have shown in the present study a negative influence of PE and DAG activation of these PKC isoforms on the insulin-stimulated AC system. If the calphostin C inhibitory effect were directed only to PE-sensitive PKC isoforms that exercise a negative influence on the AC system, then the AC-stimulating action of insulin would remain unchanged. Consequently, the inhibitory action of calphostin C may also be extended to PE-insensitive isoforms of PKC. Among them, the first candidate that may be involved in the insulin ACSM is PKCζ, which is activated by the products of the PI3-K reaction triggered by insulin [58] and is present in almost all tissues [18]. Reinforcing our argument are our data on wortmannin inhibition of insulin signal transduction via the AC system (Table 2). Taking into account the ability of PKC, PKCζ, in particular, to phosphorylate both G_s-protein and AC [55,56], PKCζ should be located upstream of G_s-protein in the AC-signaling cascade. The supposition that growth factors, including insulin, can regulate AC activity (specifically AC isoform V) through PKCζ was also voiced by other authors [56]. Taking into account that our data concerning the involvement of PKCζ in the ACSM of insulin action are indirect, we are in the process of planning a study using specific antibodies for PKCζ or specific inhibitor of the enzyme, which would allow us to obtain the necessary confirmation.

All the data obtained lead to the following conclusion. The ACSM of insulin action in all probability includes, in addition to previously identified functional blocks such as receptor tyrosine kinase, G_s-protein, and AC [10,11], other components such as G_i-protein, PI3-K, and PKCζ. On the basis of our data and those in the literature, the following insulin signal transduction cascade via the AC system may be suggested. In the first stage, insulin-induced activation of receptor tyrosine kinase leads to activation of G_i-protein accompanied by dissociation and liberation of its α- and $\beta\gamma$-subunits. There is theoretical and experimental evidence in favor of a direct interaction of insulin receptor with $G_{i/o}$-proteins [65–67] as well as of participation of G_i-protein in the hormone action [68]. In the second stage, $\beta\gamma$-dimer activates PI3-K, which catalyzes phosphoinositide phosphorylation followed by the formation of 3-phosphoinositides [56,69]. In the third stage, the lipid messengers activate atypical PKC isoforms including PKCζ [58]. In the final stage, PKCζ phosphorylates and, as a result, activates alternatively either G_s-protein or directly AC (e.g. AC isoform V).

The total result of the above events is stimulation of AC activity and intensification of cAMP formation. Thus, the discovery and deciphering of a novel signaling mechanism of insulin action involving the AC system which had been denied in the literature will add missing knowledge to the current picture of the insulin-signaling network concerning the role of cAMP in the regulatory effects of insulin and related peptides. There are grounds to believe that this ACSM is involved, first, in the action of insulin and other insulin superfamily peptides [10–12] and second, according to our hypothesis [10,11], in the mitogenic rather than metabolic action of these peptides. Data in the literature concerning the key role of PI3-K and PKCζ in mitogenic signaling [70,71] serve as additional evidence in favor of our hypothesis.

In conclusion we tried, on the basis of the literature and our

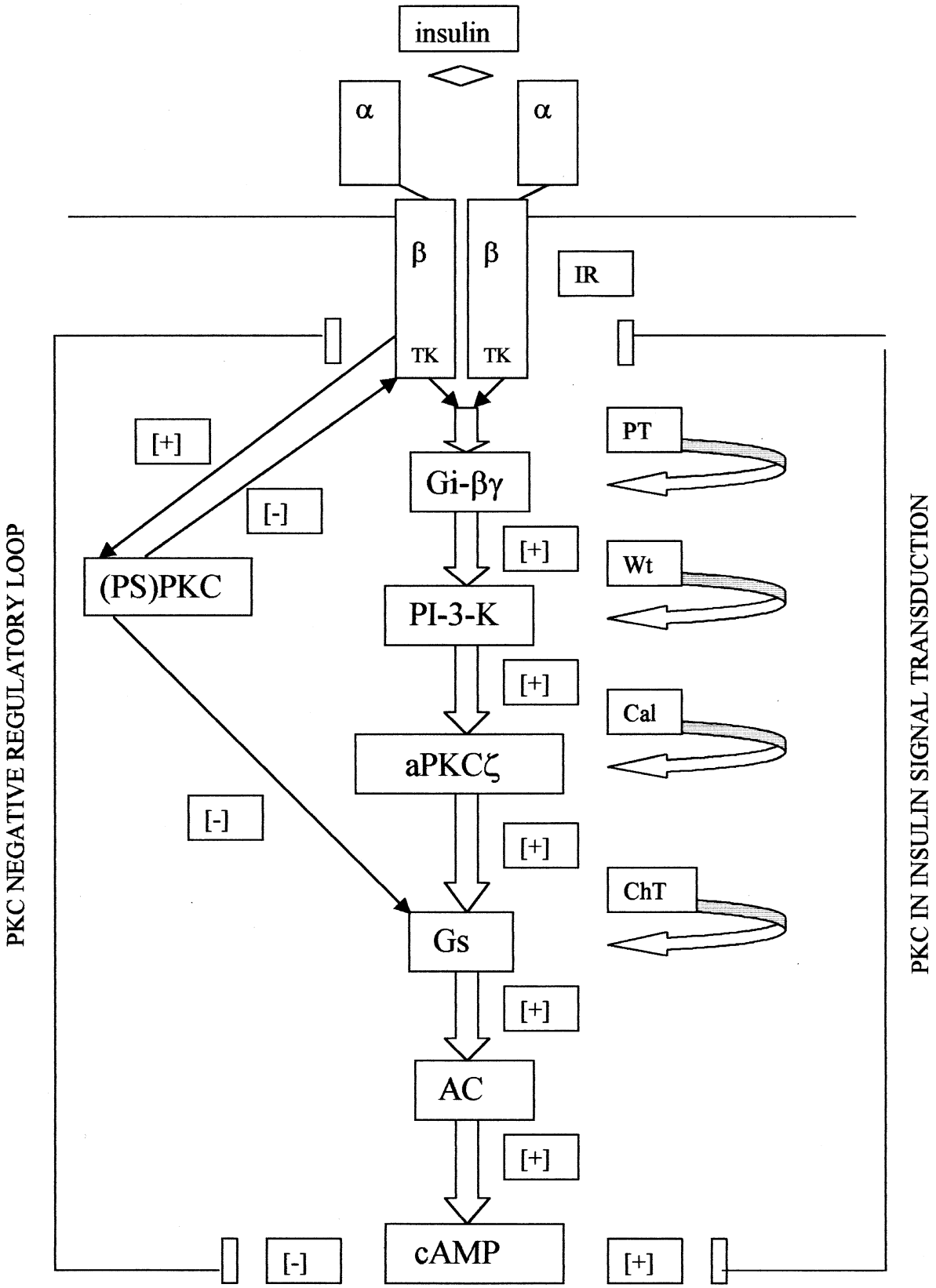

Fig. 7. Hypothetical scheme of the dual role of protein kinase C in transduction of insulin signal via the adenylyl cyclase signaling mechanism. The spatial arrangement of the ACSM is represented as a simplified linear chain of signaling blocks and events. In reality, under the condition of insulin action in a cell, the ACSM components interact with each other in the plasma membrane and/or premembrane regions where they form temporary functional complexes. IR, insulin receptor; α and β, subunits of IR; TK, tyrosine kinase domain of IR; (PS)PKC, phorbol-sensitive PKC; aPKCζ, atypical phorbol-insensitive PKC isoform; Wt, wortmannin; Cal, calphostin C; [−], inhibition; [+], activation.

data, to build a hypothetical scheme showing a dual role of the PKC system in insulin signal transduction via the ACSM (Fig. 7). The regulatory role is to secure negative regulation (modulation) of this signaling system via the feedback loop: insulin, through receptor tyrosine kinase phosphorylation, activates conventional PE- and DAG-sensitive PKC isoforms, which in turn phosphorylate the insulin receptor and G_s-protein at serine/threonine, reducing their signal transduction functions. This regulatory mechanism is neither hormone- nor receptor-specific, since it operates when the AC system is regulated by other hormones (e.g. biogenic amines) via serpentine receptors. It very likely represents the universal mechanism of desensitization of the hormone-regulated AC system that blocks signal transmission [46,72]. In the case of the insulin-reactive AC system, this mechanism may be the cause of cell insulin resistance [26,50,73]. The second role of PKC in the insulin-regulated AC system, which we refer to as transductory, is performed by atypical isoforms of PKC, apparently PKCζ. It is a direct participation of PKC in transduction of the insulin signal from the receptor to AC.

A similar idea about the dual isoform-specific role of PKC in insulin signaling was put forward by Formisano *et al*. (1998) [52], who showed its regulatory role on the initial stages of insulin action: insulin-inducible activity of receptor tyrosine kinase, IRS-signaling functions, and intracellular routing of insulin receptor. The transductory role of PKC is manifested in its participation in transmission of the insulin signal to the systems of glucose transport and metabolism.

Acknowledgments

We express our thanks to Inga Menina for linguistic assistance. This research was supported in part by the Russian Foundation for Basic Research (Grants 96-15-97894, 97-04-48961, and 00-04-49465).

References

[1] Bell JD, Branton LL. Multiple effects of phorbol esters on hormone-sensitive adenylate cyclase activity in S49 lymphoma cells. Am J Physiol 1987;252:E783–9.
[2] Dixon BS, Breckon R, Burke C, Anderson RJ. Phorbol esters inhibit adenylate cyclase activity in cultured collecting tubular cells. Am J Physiol 1988;254:183–91.
[3] Morimoto BH, Koshland DE. Conditional activation of cAMP signal transduction by protein kinase C. The effect of phorbol esters on adenylyl cyclase in permeabilized and intact cells. J Biol Chem 1994;269:4065–9.
[4] Carmena MJ, Garcia-Paramio P, Solano RM, Prieto JC. Protein kinase C regulation of the adenylyl cyclase system in rat prostatic epithelium. Prostate 1995;27:204–11.
[5] Karl PJ, Divald A. Protein kinase C in cultured human placental trophoblasts: identification of isoforms and role in cAMP signalling. Biochem J 1996;320:831–6.
[6] Kawabe J, Iwami G, Ebina T, Ohno S, Katada T, Ueda Y, Homcy CJ, Ishikawa Y. Differential activation of adenylyl cyclase by protein kinase C isoenzymes. J Biol Chem 1994;269:16554–8.
[7] Nandi J, Bosche MC, Levine RA. Effects of phorbol ester and isoquinoline sulfoamines on rabbit parietal cell function. J Pharmacol Exp Ther 1996;279:97–105.
[8] Ohno S. The distinct biological potential of PKC isotypes. In: Parker PJ, Dekker LV, editors. Protein kinase C. Heidelberg: Springer-Verlag, 1997. p. 75–95.
[9] Houslay MD, Milligan G. Tailoring cAMP signalling responses through isoform multiplicity. Trends Biochem Sci 1997;22:217–24.
[10] Pertseva MN, Plesneva SA, Shpakov AO, Rusakov YI, Kuznetsova LA. Involvement of the adenylyl cyclase signaling system in the action of insulin and mollusc insulin-like peptide. Comp Biochem Physiol B Biochem Mol Biol 1995;112:689–95.
[11] Pertseva MN, Plesneva SA, Kuznetsova LA, Shpakov AO, Derkach KV. On the tyrosine kinase mechanism of the novel effect of insulin and insulin-like growth factor 1. Stimulation of the adenylyl cyclase system in muscle tissue. Biochem Pharmacol 1996;52:1867–74.
[12] Kuznetsova L, Plesneva S, Derjabina N, Omeljaniuk E, Pertseva M. On the mechanism of relaxin action: the involvement of adenylyl cyclase signalling system. Regul Pept 1999;80:33–9.
[13] Farese RV, Standaert ML, Arnold T, Yu B, Ishizuka T, Hoffman I, Via M, Cooper DR. The role of protein kinase C in insulin action. Cell Signal 1992;4:133–43.
[14] Bandyopadhyay G, Standaert ML, Zhao LM, Yu B, Avignon A, Galloway L, Karnam P, Moscat J, Farese RV. Activation of protein kinase C (α, β, and ζ) by insulin in 3T3/L1 cells. Transfection studies suggest a role for PKCζ in glucose transport. J Biol Chem 1997;272:2551–8.
[15] Lai H-L, Yang TH, Messing RO, Ching Y-H, Lin S-C, Chern Y. Protein kinase C inhibits adenylyl cyclase type VI activity during desensitization of the A2α-adenosine receptor-mediated cAMP response. J Biol Chem 1997;272:4970–7.
[16] Plesneva SA, Kuznetsova LA, Shpakov AO, Pertseva MN. Adenylyl cyclase signalling mechanism is involved in the action of insulin *in vivo* in tissues and cells of vertebrates and invertebrates. Abstracts of the Fifth International Congress of Comparative Physiology and Biochemistry (ICCPB 99), Calgary, Alberta, Canada, 23–28 August 1999, S74, 1999.
[17] Standaert ML, Bandyopadhyay G, Zhou X, Galloway L, Farese RV. Insulin stimulates phospholipase D-dependent phosphatidylcholine hydrolysis, Rho translocation, *de novo* phospholipid synthesis, and diacylglycerol/protein kinase C signaling in L6 myotubes. Endocrinology 1996;137:3014–20.
[18] Liu WS, Heckman CA. The sevenfold way of PKC regulation. Cell Signal 1998;10:529–42.
[19] Toker A, Cantley LC. Signalling through the lipid products of phosphoinositide-3-OH-kinase. Nature 1997;387:673–6.
[20] Mothe I, Delahaye L, Filloux C, Pons S, White MF, Van Obberghen E. Interaction of wild type and dominant-negative p55PIK regulatory subunit of phosphadylinositol 3-kinase with insulin-like growth factor-1 signaling proteins. Mol Endocrinol 1997;11:1911–23.
[21] Shpakov AO. Structural-functional characteristics of heterodimer phosphatidylinositol 3-kinase and molecular mechanisms of its coupling with other components of the signalling system. Tsitologiia 1999;41:975–91. Russian.
[22] Taussing R, Gilman AG. Mammalian membrane-bound adenylyl cyclases. J Biol Chem 1995;270:1–41.
[23] Shpakov AO, Pertseva MN. Structural-functional characteristics of G-protein β- and γ-subunits and molecular mechanisms of their coupling with other components of the signal transduction system. J Evol Biochem Physiol 1997;33:582–94.
[24] Milligan G. Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding properties. Biochem J 1988;255:1–13.
[25] Nixon JS. The biology of protein kinase C inhibitors. In: Parker PJ, Dekker LV, editors. Protein kinase C. Heidelberg: Springer-Verlag, 1997. p. 205–29.

[26] Berti L, Mosthaf L, Kroder G, Kellerer M, Tippmer S, Mushack J, Seffer E, Seedorf K, Haring H. Glucose-induced translocation of protein kinase C isoforms in rat-1 fibroblasts is paralleled by inhibition of the insulin receptor tyrosine kinase. J Biol Chem 1994;269: 3381–6.
[27] McKenna JP, Williams JM, Hanson PJ. The alpha isoform of protein kinase C inhibits histamine-stimulated adenylate cyclase activity in a particulate fraction of the human gastric cancer cell line HGT-1. Inflamm Res 1995;44:66–9.
[28] Yamada K, Avignon A, Standaert ML, Cooper DR, Spencer B, Farese RV. Effects of insulin on the translocation of protein kinase C-theta and other protein kinase C isoforms in rat skeletal muscles. Biochem J 1995;308:177–80.
[29] Avignon A, Standaert ML, Yamada K, Mischak H, Spencer B, Farese RV. Insulin increases mRNA of protein kinase C-α and -β in rat adipocytes and protein kinase C-α, β and θ in rat skeletal muscle. Biochem J 1995;308:181–7.
[30] Newton AC. Regulation of protein kinase C by cofactors. In: Parker PJ, Dekker LV, editors. Protein kinase C. Heidelberg: Springer-Verlag, 1997. p. 25–45.
[31] Yano H, Nakanishi S, Kimura K, Hanai N, Saitoh Y, Fukui Y, Nonomura Y, Matsuda Y. Inhibition of histamine secretion by wortmannin through the blockage of phosphatidylinositol 3-kinase in RBL-2H3 cells. J Biol Chem 1993;268:25846–56.
[32] Reisine T. Pertussis toxin in the analysis of receptor mechanisms. Biochem Pharmacol 1990;39:1499–504.
[33] Kidwai AM, Radcliffe MA, Lee EY, Daniel EE. Isolation and properties of skeletal muscle membranes. Biochim Biophys Acta 1973; 298:593–607.
[34] Pertseva MN, Kuznetsova LA, Mazina TI, Plesneva SA. On the role of guanyl nucleotides in adenylate cyclase system of embryonic skeletal muscle. Biochem Int 1983;6:789–97.
[35] Salomon Y, Londs C, Rodbell M. A highly sensitive adenylate cyclase assay. Anal Biochem 1974;58:541–8.
[36] White AA. Separation and purification of cyclic nucleotides by alumina column chromatography. Methods Enzymol 1974;380:41–6.
[37] Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;195:265–75.
[38] Srinivasan M, Begum N. Stimulation of protein phosphatase-1 activity by phorbol esters. Evaluation of the regulatory role of protein kinase C in insulin action. J Biol Chem 1994;269:16662–7.
[39] Pertseva MN, Kuznetsova LA, Plesneva SA, Grishin AV, Panchenko MP. β-Agonist-induced inhibitory-guanine-nucleotide-binding regulatory protein coupling to adenylate cyclase in mollusc *Anodonta cygnea* foot muscle sarcolemma. Eur J Biochem 1992;210:279–86.
[40] Pertseva MN. The evolution of hormonal signalling systems. Comp Biochem Physiol A 1991;100:775–87.
[41] Pepio AM, Fan X, Sossin WS. The role of C2 domains in Ca^{2+}-activated and Ca^{2+}-independent protein kinase Cs in aplysia. J Biol Chem 1998;273:19040–8.
[42] Jacobowitz O, Chen J, Premont RT, Iyengar R. Stimulation of specific types of Gs-stimulated adenylyl cyclases by phorbol ester treatment. J Biol Chem 1993;268:3829–32.
[43] Tamura T, Takahashi H, Iizuka H. Protein kinase C-dependent modulation of stimulatory guanine nucleotide binding protein of fetal rat skin keratinocytes. Arch Dermatol Res 1996;288:24–30.
[44] Zimmermann G, Taussig R. Protein kinase C alters the responsiveness of adenylyl cyclases to G protein alpha and beta/gamma subunits. J Biol Chem 1996;271:27161–6.
[45] Sagi-Eisenberg R. GTP-binding proteins as possible targets for protein kinase C action. Trends Biochem Sci 1989;14:355–7.
[46] Aragay AM, Quick MW. Functional regulation of Galpha16 by protein kinase C. J Biol Chem 1999;274:4807–15.
[47] Takayama S, White MF, Kahn CR. Phorbol ester-induced serine phosphorylation of the insulin receptor decreases its tyrosine kinase activity. J Biol Chem 1988;263:3440–7.
[48] Danielson AG, Liu F, Hosomi J, Shii K, Roth RA. Activation of protein kinase C alpha inhibits signaling by members of the insulin receptor family. J Biol Chem 1995;270:21600–5.
[49] Yoshimura M, Cooper DM. Cloning and expression of a Ca^{2+}-inhibitable adenylyl cyclase from NCB-20 cells. Proc Natl Acad Sci USA 1992;89:6716–20.
[50] Chen KS, Heydrick SJ, Brown ML, Friel JC, Ruderman NB. Insulin increases a biochemically distinct pool of diacylglycerol in the rat soleus muscle. Am J Physiol 1994;266:E479–85.
[51] Liu F, Roth RA. Insulin-stimulated tyrosine phosphorylation on protein kinase C alpha: evidence for direct interaction of the insulin receptor and protein kinase C in cells. Biochem Biophys Res Commun 1994;200:1570–7.
[52] Formisano P, Oriente F, Miele C, Caruso M, Auricchio R, Vigliotta G, Condorelli G, Beguinot F. In NIH-3T3 fibroblasts, insulin receptor interaction with specific protein kinase C isoforms controls receptor intracellular routing. J Biol Chem 1998;273:11197–202.
[53] Sossin WS, Chen CS, Toker A. Stimulation of an insulin receptor activates and down-regulates the Ca^{2+}-independent protein kinase C, Apl II, through a wortmannin-sensitive signaling pathway in Aplysia. J Neurochem 1996;67:220–8.
[54] Krupinski J, Lehman TC, Frankenfield CD, Zwaagstra JC, Watson PA. Molecular diversity in the adenylyl cyclases. J Biol Chem 1992; 267:24858–62.
[55] Kawabe J, Ebina T, Toya Y, Oka N, Schwencke C, Duzic E, Ishikawa Y. Regulation of type V adenylyl cyclase by PMA- sensitive and -insensitive protein kinase C isoenzymes in intact cells. FEBS Lett 1996;384:273–6.
[56] Ishikawa Y, Homcy CJ. The adenylyl cyclases as integrators of transmembrane signal transduction. Circ Res 1997;80:297–304.
[57] Lustig KD, Conklin BR, Herzmark P, Taussig R, Bourne HR. Type II adenylylcyclase integrates coincident signals from Gs, Gi, and Gq. J Biol Chem 1993;268:13900–5.
[58] Standaert ML, Galloway L, Karnam P, Bandyopadhyay G, Moscat J, Farese RV. Protein kinase C-zeta as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes. Potential role in glucose transport. J Biol Chem 1997;272:30075–82.
[59] Yamamoto-Honda R. Upstream mechanisms of glycogen synthase activation by insulin and insulin-like growth factor-I. J Biol Chem 1995;270:2729–34.
[60] Czech MP. PJP2 and PJP3: complex roles at the cell surface. Cell 2000;100:603–6.
[61] Wilson M, Burt AR, Milligan G, Anderson NG. Wortmannin-sensitive activation of p70s6k by endogenous and heterologously expressed Gi-coupled receptors. J Biol Chem 1996;271:8537–40.
[62] Mhaouty-Kodja S, Bouet-Alard R, Limon-Boulez G, Maltier JP, Legrand C. Molecular diversity of adenylyl cyclases in human and rat myometrium. Correlation with global adenylyl cyclase activity during mid- and term pregnancy. J Biol Chem 1997;272:31100–6.
[63] Marjamaki A, Sato M, Bouet-Alard R, Yang Q, Limon-Boulez I, Legrand C, Lanier SM. Factors determining the specificity of signal transduction by guanine nucleotide-binding protein-coupled receptors. Integration of stimulatory and inhibitory input to the effector adenylyl cyclase. J Biol Chem 1997;272:16466–73.
[64] Xu J, Clark AF. A three-dimensional collagen lattice induces protein kinase ζ activity: role in α_2 integrin and collagenase mRNA expression. J Cell Biol 1997;136:473–83.
[65] Okamoto J, Okamoto T, Murayama Y, Hayashi Y, Ogata E, Nishimoto I. GTP-binding protein-activator sequences in the insulin receptor. FEBS Lett 1993;334:143–8.
[66] Shpakov AO. The homology of the primary structure of the third cytoplasmic domains in rhodopsin-type receptors and of the cyplasmic tail in the β-subunit of the insulin receptor. Tsitologiia 1996;38: 1179–90.
[67] O'Brien RM, Houslay MD, Milligan G, Siddle K. The insulin receptor tyrosyl kinase phosphorylates holomeric forms of the

guanine nucleotide regulatory proteins G_i and G_o. FEBS Lett 1987;212:281–8.
[68] Luft FC. G-proteins and insulin signaling. J Mol Med 1997;75:233–5.
[69] Sharma PM, Egawa K, Huang Y, Martin JL, Huvar I, Boss GR, Olefsky JM. Inhibition of phosphatidylinositol 3-kinase activity by adenovirus-mediated gene transfer and its effect on insulin action. J Biol Chem 1998;273:18528–37.
[70] Berra E, Diaz-Meco MT, Dominguez J, Municio MM, Sanz L, Lozano J, Chapkin RS, Moscat J. Protein kinase C zeta isoform is critical for mitogenic signal transduction. Cell 1993;74:555–63.
[71] Gupta BB. Mechanism of insulin action. Curr Sci 1997;73:993–1003.
[72] Diviani D, Lattion AL, Cotecchia S. Characterization of the phosphorylation sites involved in G protein-coupled receptor kinase- and protein kinase C-mediated desensitization of the alpha1B-adrenergic receptor. J Biol Chem 1997;272:28712–6.
[73] de Vente JE, Carey JO, Bryant WO, Pettit GJ, Ways DK. Transcriptional regulation of insulin receptor substrate 1 by protein kinase C. J Biol Chem 1996;271:32276–80.