Biochemistry (Moscow), Vol. 67, No. 3, 2002, pp. 335-342. Translated from Biokhimiya, Vol. 67, No. 3, 2002, pp. 403-412.

Study of the Functional Organization of a Novel Adenylate Cyclase Signaling Mechanism of Insulin Action

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Received February 12, 2001
Revision received May 15, 2001

Abstract—In this study we continued decoding the adenylate cyclase signaling mechanism that underlies the effect of insulin and related peptides. We show for the first time that insulin signal transduction via an adenylate cyclase signaling mechanism, which is attended by adenylate cyclase activation, is blocked in the muscle tissues of the rat and the mollusk *Anodonta cygnea* in the presence of: 1) pertussis toxin, which impairs the action of the inhibitory GTP-binding protein (G_i); 2) wortmannin, a specific blocker of phosphatidylinositol 3-kinase; and 3) calphostin C, an inhibitor of different isoforms of protein kinase C. The treatment of sarcolemmal membrane fraction with cholera toxin increases basal adenylate cyclase activity and decreases the sensitivity of the enzyme to insulin. We suggest that the stimulating effect of insulin on adenylate cyclase involves the following stages of hormonal signal transduction cascade: receptor tyrosine kinase → G_i protein ($\beta\gamma$) → phosphatidylinositol 3-kinase → protein kinase C (ζ?) → G_s protein → adenylate cyclase → cAMP.

Key words: insulin, adenylate cyclase, protein kinase C, phosphatidylinositol 3-kinase, hormonal signaling system, bacterial toxin, wortmannin, calphostin C

A hormone-sensitive adenylate cyclase signaling system plays a key role in hormonal signal transduction in the cells of animals of different phylogenetic levels. This system consists of three main components: a receptor that recognizes and specifically binds the hormone molecule, a heterotrimeric GTP-binding protein (G protein) that mediates signal transduction to the third, catalytic component of adenylate cyclase signaling system, and the enzyme *per se* (i.e., adenylate cyclase). It is believed that an adenylate cyclase signaling system underlies the effect of hormones acting via serpentine-type receptors containing seven transmembrane domains. However, data on the involvement of the adenylate cyclase signaling system in the mechanisms underlying the regulatory effect of hormones, whose effect is mediated by tyrosine kinase receptors containing the tyrosine kinase domain, have been absent from the literature until recently. The results of our studies on the involvement of adenylate cyclase signaling system in the regulatory effect of insulin and related peptides—insulin-like growth factor I (IGF-I), relaxin, and insulin-like substance of the mollusk *Anodonta cygnea*—for the first time showed that the effects of these compounds may be realized via the signaling cascade comprised of the receptor tyrosine kinase, G_s protein, and adenylate cyclase [1-5].

In this study, we continued decoding the adenylate cyclase signaling mechanism underlying the effect of peptides belonging to the insulin superfamily. With regard for the stimulating effect of insulin on phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) reported in literature and the association between the adenylate cyclase and PKC systems, in this study we checked the possibility of involvement of the aforementioned enzyme systems and heterotrimeric G proteins in the adenylate cyclase signal mechanism of insulin action.

The study was performed on rat skeletal muscles and smooth muscles of the mollusk *A. cygnea* (the sarcolemmal membrane fraction), in which we first discovered the adenylate cyclase signal mechanism of action of the insulin superfamily peptides [1-4].

Abbreviations: IGF-I) insulin-like growth factor I; PI3K) phosphatidylinositol 3-kinase; PKC) protein kinase C; PtdIns-3,4,5-P_3) phosphatidylinositol-3,4,5-trisphosphate; PT) pertussis toxin; CT) cholera toxin.

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As shown earlier, the sequence of signaling events associated with the insulin effect on PI3K and PKC systems may be different. The first variant implies interaction with endogenous protein substrates of the insulin receptor, which, in turn, binds with the SH2 domain-containing proteins (the PI3K regulatory subunit, phosphotyrosine-specific phosphatase, GRB2 adapter protein, etc.) thereby regulating their activity [6, 7]. Activation of PI3K induces 3-phosphoinositide production and simultaneously triggers several signal cascades in the cell [8, 9]. One of these cascades, with inositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3) as a secondary messenger, stimulates the activity of atypical phorbol-resistant PKC isoforms (PKCζ in particular) [10-14]. The second variant implies PI3K activity regulation by the activated receptor via signaling pathways that do not involve the endogenous protein substrates of the insulin receptor. In this case, the regulatory subunit of the enzyme directly interacts with the receptor molecule (presumably, with its C-terminal domain) [15-17]. Finally, PI3K can be activated as a result of interaction of the enzyme with the $\beta\gamma$ dimer of heterotrimeric G proteins (primarily, the G_i protein as the main donor of $\beta\gamma$ dimer) [9, 18-24].

To determine the stage at which PI3K participates in the insulin-triggered adenylate cyclase signaling cascade, we studied the effect of wortmannin, a specific inhibitor of PI3K. Wortmannin (a metabolite of fungi belonging to the genus *Penicillium*) is a potent selective and irreversible inhibitor of PI3K [25]. It blocks PI3K activity through direct interaction with the enzyme catalytic subunit. Wortmannin can also inhibit other kinases (e.g., myosin light chain kinase and phosphoinositide 4-kinase) at concentrations 1000-fold greater than those required for PI3K inhibition.

Given that G proteins are involved in insulin signaling [1-4, 26-31] and with regard for the effect of $\beta\gamma$ dimer on hormone-dependent PI3K, we studied the role of stimulating and inhibitory heterotrimeric G proteins in the insulin-activated adenylate cyclase signaling. Pertussis toxin (PT) and cholera toxin (CT) were used for functional identification of G_i and G_s proteins, respectively [32, 33].

To determine whether PKC is involved in the adenylate cyclase signaling mechanism, we studied the effect of calphostin, a highly selective inhibitor of different PKC isoforms (including atypical forms) in insulin signaling via the adenylate cyclase signaling system. Inhibition of PKC activity by calphostin is due to irreversible inactivation of the enzyme through strong binding of a photoactivated form of the inhibitor with the cysteine-rich Zn^{2+}-binding sites of the PKC regulatory domain [34].

MATERIALS AND METHODS

The study was performed on sarcolemmal membrane fractions of foot smooth muscles of the freshwater bivalve mollusk *Anodonta cygnea* and skeletal muscles of hind legs of the rat *Rattus norvegicus*. Membrane fractions were isolated as described by Kidwai et al. [35]. Each fraction was obtained from 25-30 mollusks and 6-8 rats. We attained five- to eightfold enrichment of the membrane fraction, as assessed by adenylate cyclase and 5′-nucleotidase activity.

The activity of adenylate cyclase (cyclizing ATP pyrophosphatase, EC 4.6.1.1) was determined by the method of Salomon et al. [36] with some modifications. The incubation medium (final volume 50 μl) contained 50 mM Tris-HCl (pH 7.5), 0.1 mM ATP, 1 mM cAMP, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 5 mM $MgCl_2$, and [α-^{32}P]ATP (1-2 μCi). The reaction was started by the addition of 15-20 μg protein to the incubation medium. After incubation for 2.5 h (in the case of insulin and IGF-I) or 10 min (in the case of isoproterenol) at 30°C (mollusks) or 37°C (rats), the reaction was stopped by the addition of 100 μl 0.5 M HCl. The specimens were heated in a boiling water bath for 7 min. To neutralize the acid, 100 μl of 1.5 M imidazole was added to each specimen. Cyclic AMP formed during the reaction was determined by the method of White [37] using chromatographic separation of nucleotides on a column with aluminum oxide. Then cAMP was eluted with 8 ml of 10 mM imidazole-HCl buffer (pH 7.4). The eluates containing radioactively labeled cAMP were collected and assayed for radioactivity by the Cherenkov method on an LKB 1209/1215 Rackbeta radioactivity counter (LKB, Sweden). Adenylate cyclase activity was expressed in pmol cAMP/min per mg protein.

The effects of insulin, IGF-I, toxins, calphostin, and wortmannin on adenylate cyclase activity were studied *in vitro*. Insulin was dissolved in 0.01 M HCl, and calphostin C was dissolved in dimethyl sulfoxide. The stock solutions were diluted with Tris-HCl (pH 7.5) to the required concentrations immediately before use. After preincubation of membrane fraction with calphostin C for 10 min in the light (or with wortmannin for 5 min), insulin or IGF-I (at concentrations that maximally stimulate adenylate cyclase) was added to the mixture [1-3]. The duration of insulin action was 2.5 min, which is necessary and sufficient for displaying its maximal effect on adenylate cyclase. The specimens that were similarly treated but did not contain hormones, toxins, calphostin C, or wortmannin served as a control.

ADP-ribosylation with bacterial toxins was performed as follows. The membrane fraction (with protein content approximately 0.95-1.00 mg/ml) was incubated at 30°C (mollusks) or 37°C (rats) for 45 min in the presence or absence of the toxins (10 μg/ml PT or 100 μg/ml CT). The incubation medium (400 μl) contained 50 mM Tris-HCl buffer (pH 7.8), 2 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol, 0.1 mM NAD^+, 1 mM $NADP^+$, 0.1 mM GTP (in the case of PT) or 5′-guanylylimidodiphosphate (Gpp[NH]p) (in the case of CT), 1 mM

ATP, and 10 mM thymidine. The toxins were preliminarily activated by incubating at 37°C for 15 min in the presence of dithiothreitol and ATP. After ADP-ribosylation, the suspension was diluted into 5 ml of cold 50 mM Tris-HCl buffer (pH 7.5), and the specimens were centrifuged at 100,000*g* for 30 min. The pellet was resuspended in the same buffer and immediately used for assaying the adenylate cyclase activity.

Creatine phosphate, rabbit muscle creatine phosphokinase (EC 2.7.3.2), ATP, GTP, Gpp[NH]p, cAMP, dithiothreitol, imidazole, EDTA, NAD, NADP, thymidine, wortmannin (from the fungus *Penicillium wortmannii*), pertussis toxin (from *Bordetella pertussis* bacterial culture), cholera toxin (from the bacterium *Vibrio cholerae*), calphostin C (from *Cladosporium cladosporioides*), and aluminum oxide for column chromatography were obtained from Sigma (USA). [α-^{32}P]ATP (4 Ci/mmol) was purchased from Isotope (St. Petersburg, Russia). Mammalian insulin (24 IU) was received from Lilly Co. (USA), and recombinant human IGF-I was from Amersham (UK).

The results were statistically processed using the analysis of variance (ANOVA) test (USA). Each experiment was made in triplicate. The data were expressed as M $\pm$ SEM (of several independent experiments). The differences between the control and the specimens treated with hormones, toxins, calphostin, and wortmannin were regarded as statistically significant at $p < 0.05$.

RESULTS

We first studied the influence of a specific PI3K inhibitor, wortmannin, on adenylate cyclase-stimulating effect of insulin and IGF-I in mollusk and rat muscles.

After preincubation of mollusk sarcolemmal membranes with wortmannin (10^{-10}-10^{-7} M), adenylate cyclase basal activity (which in the absence of the inhibitor was 18.1 pmol cAMP/min per mg protein) somewhat decreased (Fig. 1). Wortmannin reduced the adenylate cyclase-stimulating effect of insulin (10^{-8} M) in mollusk muscles in a concentration-dependent manner. At concentrations of 10^{-9}-10^{-7} M, wortmannin completely blocked this effect of insulin (Fig. 1). A qualitatively similar picture was observed when studying the influence of wortmannin on adenylate cyclase-stimulating effect of IGF-I (10^{-9} M). The only difference was the effectiveness of wortmannin inhibitory action on hormonal stimulation of adenylate cyclase: in the case of IGF-I the inhibitor effect was not as pronounced as in the case of insulin, and the adenylate cyclase-stimulating effect of wortmannin was partially retained (Fig. 1). A similar pattern of inhibition of the insulin-stimulated activity of adenylate cyclase by wortmannin (10^{-9}-10^{-7} M) was observed in rat sarcolemmal membranes (Fig. 2). Basal activity of adenylate cyclase (30.9 pmol cAMP/min per mg protein) only slightly decreased at wortmannin concentrations of 10^{-8}-10^{-7} M. These results indicate that PI3K is involved in the adenylate cyclase signal mechanism of action of insulin superfamily peptides.

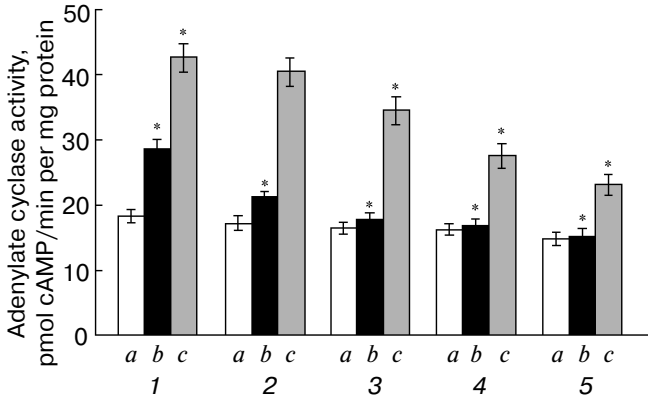

Fig. 1. Inhibitory effect of wortmannin (10^{-10}-10^{-7} M) on the stimulation of adenylate cyclase activity in sarcolemmal membranes of the mollusk *A. cygnea* by insulin (10^{-8} M) (b) and IGF-I (10^{-9} M) (c) compared to control (without hormone) (a): *1*) without wortmannin; *2-5*) in the presence of 10^{-10} (*2*), 10^{-9} (*3*), 10^{-8} (*4*), and 10^{-7} M (*5*) wortmannin; * $p < 0.05$.

To check the hormonal specificity of the wortmannin effect, we studied its influence on adenylate cyclase activity stimulated by isoproterenol, whose action is mediated by β-adrenergic receptors of serpentine type and is not associated with the PI3K signaling system. We found that wortmannin had no significant effect on adenylate cyclase activation by isoproterenol in rat sarcolemmal membranes (Fig. 2). This is indicative of the specificity of

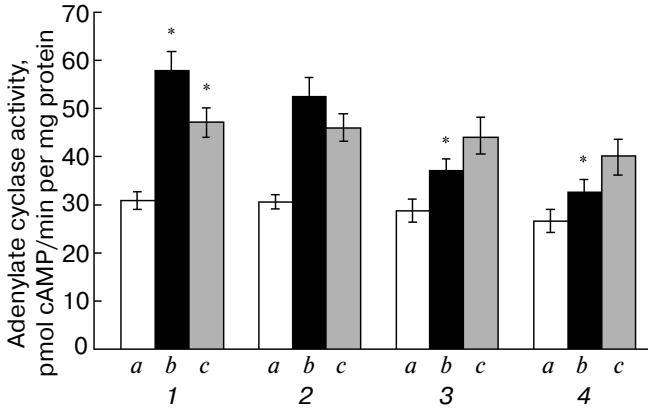

Fig. 2. Inhibitory effect of wortmannin (10^{-9}-10^{-7} M) on the stimulation of adenylate cyclase activity in sarcolemmal membranes of rat skeletal muscles by insulin (10^{-8} M) (b) and isoproterenol (10^{-5} M) (c) compared to control (without hormone) (a): *1*) without wortmannin; *2-4*) in the presence of 10^{-9} (*2*), 10^{-8} (*3*), and 10^{-7} M (*4*) wortmannin; * $p < 0.05$.

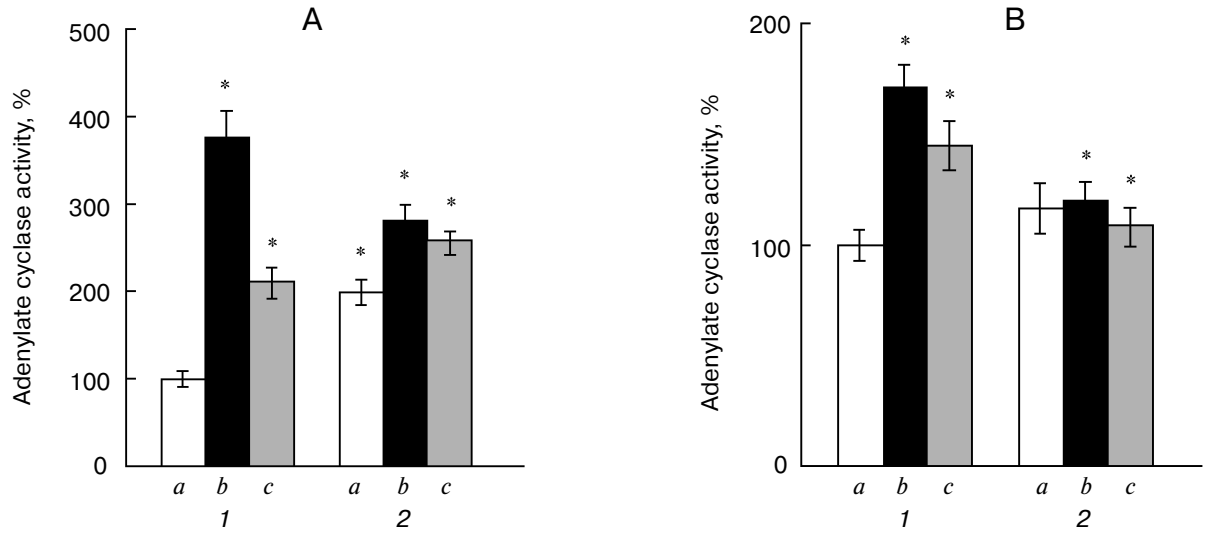

Fig. 3. Effect of pertussis toxin (PT) on basal adenylate cyclase activity (a) and that stimulated by insulin (10^{-8} M) (b) and IGF-I (10^{-9} M) (c) in sarcolemmal membranes of the mollusk *A. cygnea* (A) and rat (B): *1*) without PT, and *2*) with PT. The ordinate shows the adenylate cyclase activity expressed in percent. Basal activity of the enzyme (29.3 and 33.5 pmol cAMP/min per mg protein for mollusk and rat adenylate cyclase, respectively) was taken as 100%. Asterisk indicates statistically significant differences between the hormone-treated specimens in the absence (*1*) and presence (*2*) of the toxin: $*p < 0.05$.

PI3K involvement in adenylate cyclase signal mechanism of action of peptides of the insulin superfamily.

Next, we studied the effect of bacterial toxins, PT and CT (which modify α subunits of G_i and G_s proteins, respectively), on the regulation of adenylate cyclase activity by the peptides of the insulin superfamily.

PT treatment of mollusk and rat sarcolemmal membranes insignificantly increased adenylate cyclase basal activity and blocked the stimulating effect of insulin (10^{-8} M) (from 282 to 8% in the mollusk and from 71 to 3% in the rat) (Fig. 3). Similar results were obtained when studying the effect of PT on the adenylate cyclase-stimu-

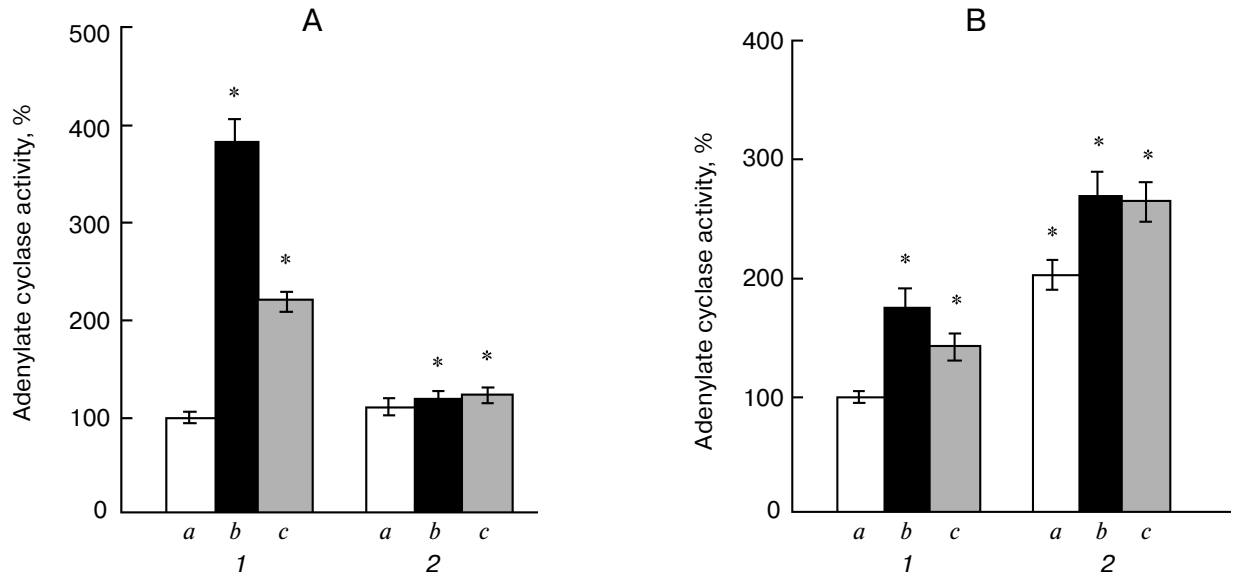

Fig. 4. Effect of cholera toxin (CT) on basal adenylate cyclase activity (a) and that stimulated with insulin (10^{-8} M) (b) and IGF-I (10^{-9} M) (c) in sarcolemmal membranes of the mollusk *A. cygnea* (A) and rat (B): *1*) without CT, and *2*) with CT. The ordinate shows the adenylate cyclase activity expressed in percent. Basal adenylate cyclase activity (31.0 and 32.8 pmol cAMP/min per mg protein for mollusk and rat enzyme, respectively) was taken as 100%. Asterisk indicates statistically significant difference between the hormone-treated specimens in the absence (*1*) and presence (*2*) of the toxin: $*p < 0.05$.

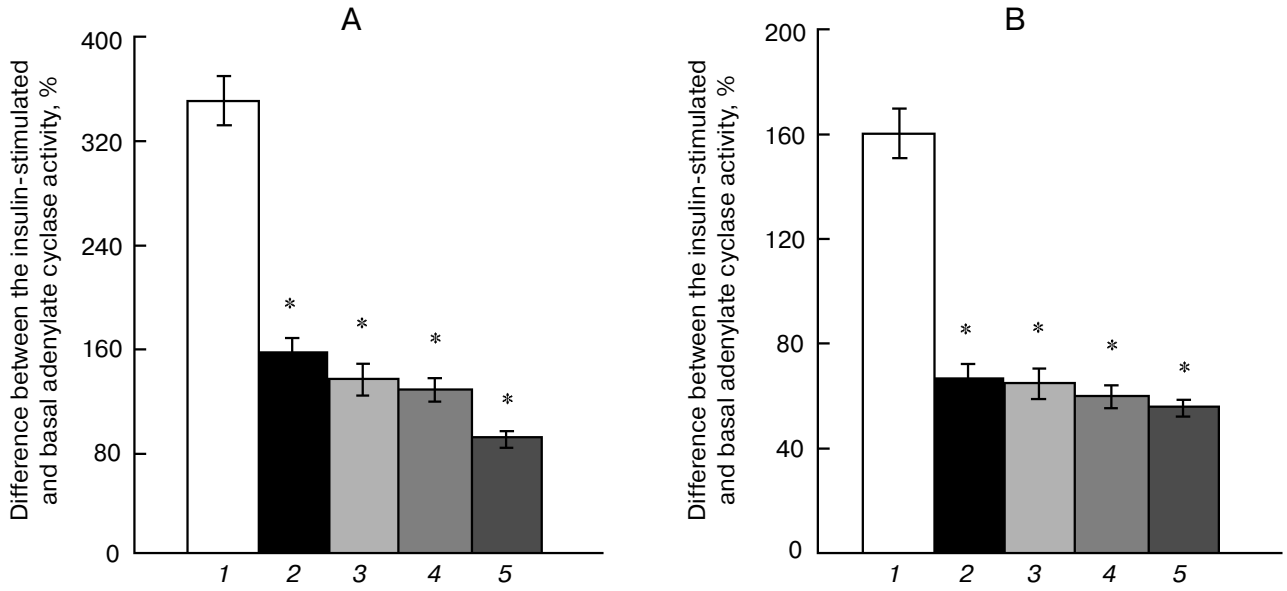

Fig. 5. The influence of calphostin C (10^{-11}-10^{-8} M) on the adenylate cyclase-stimulating effect of insulin (10^{-8} M) in sarcolemmal membranes of the mollusk *A. cygnea* (A) and rat (B): *1*) without calphostin C, and *2-5*) in the presence of 10^{-11} (*2*), 10^{-10} (*3*), 10^{-9} (*4*), and 10^{-8} M (*5*) calphostin C. The ordinate shows the difference between the insulin-stimulated and basal adenylate cyclase activity (the latter was taken as 0%). Basal adenylate cyclase activity in mollusk and rat membranes was 35.0 and 26.5 pmol cAMP/min per mg protein, respectively. Asterisks indicate statistically significant difference between the insulin-treated specimens in the absence (*1*) and presence (*2-5*) of calphostin C (*$p < 0.05$).

lating action of IGF-I (Fig. 3). It is known that the PT effect involves ADP-ribosylation of the G_i protein α subunit, which results in the loss of its functional activity [32, 33]. This is manifested, in particular, in the abolishment of the inhibitory effect of G_i protein on adenylate cyclase basal activity and in disruption of hormonal signal transduction via G_i protein-coupled receptors. In our experiments, we observed a rather unexpected suppression of adenylate cyclase-stimulating effect of insulin superfamily peptides, which may be due to the disruption of dissociation of the heterotrimeric G_i protein to the α_i subunit and βγ dimer under the action of PT. As a result, βγ dimer was not released. It is known that βγ dimer can stimulate activity of different types of PI3K (including the heterodimeric p85/p110 PI3K) [8, 18-24]. Hence, the block of dissociation of the $\alpha_i\beta\gamma$ complex by the toxin prevents the insulin-induced stimulation of PI3K activity by the βγ-dependent mechanism.

The treatment of mollusk and rat sarcolemmal fraction with CT gave a twofold increase in the adenylate cyclase basal activity (by 99 and 101%, respectively) (Fig. 4). It is known that modification with CT entails the loss of GTPase activity by α_s subunit and forces the G_s protein into a permanently activated state. For this reason, the treatment of the membrane fraction with CT stimulates adenylate cyclase catalytic activity and attenuates the regulatory effects of hormones, whose action on adenylate cyclase is mediated by G_s proteins [32, 33]. Our results are completely consistent with these data. We showed that the adenylate cyclase-stimulating effect of insulin and IGF-I was much less pronounced in the presence of CT compared to control (Fig. 4). These findings are indicative of G_s protein involvement in the adenylate cyclase-stimulating effect of insulin and related peptides, thus confirming our previous results and conclusions [1-5].

At the final stage of the study, we analyzed the influence of calphostin C, a potent and selective PKC inhibitor, on the adenylate cyclase-stimulating effect of insulin and IGF-I. Light-activated calphostin C at the concentrations of 10^{-11}-10^{-8} M increased adenylate cyclase basal activity in mollusk and rat membrane fractions *in vitro* and, conversely, decreased the insulin-stimulated adenylate cyclase activity in a concentration-dependent manner (Fig. 5). Similar results were obtained when studying the effect of calphostin C on the IGF-I-stimulated adenylate cyclase activity (data not shown). Calphostin C also had a strong inhibitory effect on adenylate cyclase activity stimulated by Gpp[NH]p alone or in combination with insulin (data not shown). This indicates that the G_s protein is the most probable PKC target.

DISCUSSION

In this study we proceeded from our previous discovery of an adenylate cyclase signaling mechanism of action of insulin and related peptides (which involves stimulat-

ing G protein [1-5]) and association between this mechanism and PKC, as shown by the concentration-dependent mode of decrease in the adenylate cyclase-stimulating effect of insulin caused by activators of classic PKC forms (diacylglycerol and phorbol ester) [38]. The study of the effect of selective inhibitors of PI3K (wortmannin) and PKC (calphostin C), as well as bacterial toxins modifying heterotrimeric G proteins, on the adenylate cyclase-stimulating effect of insulin and IGF-I was performed within the framework of further stepwise decoding the adenylate cyclase signal mechanism of action of peptides belonging to the insulin superfamily [39, 40].

The study was performed on membrane fractions isolated from muscle tissues of vertebrates (rat) and invertebrates (mollusk). We chose these membrane preparations for the following reasons. First, the main components of the adenylate cyclase signaling system, which is sensitive to insulin and other hormones (the hormonal receptor, heterotrimeric G proteins, adenylate cyclase), are associated with the plasma membrane, as follows from the activation of adenylate cyclase system in response to insulin added to membrane fractions [1, 2]. Second, it is known that membrane fractions of different cells and tissues (including muscles) in basal (non-stimulated) state contain some insulin-regulated PKC isoforms (including PKC α, β, δ, ε, and θ, as well as atypical PKC ζ) [41-44]. And, finally, a series of experimental data suggest that some PI3K isoforms are also associated with the plasma membrane and integral proteins (including the insulin receptor) [45-47].

We discovered that wortmannin, a selective inhibitor of PI3K, blocks insulin signal transduction via the adenylate cyclase system, which is suggestive of PI3K involvement in the adenylate cyclase signaling cascade. It was reported in literature that wortmannin directly binds with the PI3K catalytic subunit and suppresses the production of 3-phosphoinositides in PI3K-catalyzed reaction [24, 25], thus attenuating the activation of the atypical phorbol-insensitive PKC ζ by insulin. As a result, insulin signal transduction via other (adenylate cyclase-independent) signal pathways that ensure the metabolic (glucose transport) and growth effects of the hormone is also blocked [48, 49]. Apparently, this sequence of signal transduction is characteristic of both vertebrates and invertebrates. For example, it was shown that, in the nervous tissue of the mollusk *Alpysia californica*, insulin activation of Ca^{2+}-independent PKC is inhibited by wortmannin and is restored by the artificial analog of PI3K-catalyzed reaction, PtdIns-3,4,5-P_3 [50].

The finding that insulin signal transduction via adenylate cyclase signaling system is blocked by treatment of sarcolemmal membranes with PT provides evidence that G_i protein is involved in this process. It was shown that G_i protein-coupled receptors can stimulate the effector proteins due to $\beta\gamma$ dimer release from the heterotrimeric $\alpha_i\beta\gamma$ complex [51]. With regard for the fact that some PI3K isoforms are activated by interaction with the $\beta\gamma$ dimer of G protein [9, 18-24], we suggest PI3K may be the target for the $\beta\gamma$ dimer and mediate the adenylate cyclase-stimulating effect of insulin. Moreover, for α_2-adrenergic receptor, it was shown that activation of the receptor–G_i protein system results in the potentiation of G_s-stimulated adenylate cyclase activity (type II adenylate cyclase in particular) [52]. These data are very important in terms of the postulated G_s protein involvement in the adenylate cyclase-stimulating mechanism of action of insulin and other peptides of the insulin superfamily [1-5, 39, 40].

All these facts suggest that the following additional components—G_i protein, PI3K, and PKC (most likely, isoform ζ)—are involved in the adenylate cyclase signaling mechanism of insulin action. This assumption is confirmed by the data demonstrating that the target of activating action of insulin and other growth factors is PKC ζ, which is stimulated via the PI3K signaling pathway [49, 53, 54]. It should be noted in this connection that distinct potentiation of insulin stimulating effect on cAMP production was observed when PKC ζ and type V adenylate cyclase were co-expressed in the cell, which is indicative of interaction between these two enzymes [55]. In addition, it was reported that atypical PKC isoforms (PKC ζ in particular) can also activate another adenylate cyclase isoform (type II adenylate cyclase) [56].

These data are corroborated by the results of our experiments with calphostin C, a highly selective inhibitor of all known PKC isoforms (both phorbol-sensitive and atypical [34], including PKC ζ [57]). We showed that calphostin C blocks insulin signal transmission via adenylate cyclase signaling system, as follows from its strong inhibitory influence on the adenylate cyclase-stimulation insulin effect (Fig. 5). Because calphostin C *per se* increases basal adenylate cyclase activity, the stage of its inhibitory action on adenylate cyclase activation by insulin (namely, PKC) should be somewhere upstream in the insulin-regulated adenylate cyclase signaling cascade.

Of all known PKC isoforms, whose inhibition with calphostin C blocks insulin signal transduction via adenylate cyclase signaling system, the group of phorbol-sensitive PKC should be immediately excluded. This can be done based on our previous finding that activation of these PKC isoforms by phorbol esters and diacylglycerol represses insulin signal transduction via the adenylate cyclase signaling system [38]. If calphostin C inhibited only the phorbol-sensitive PKC isoforms, which repress adenylate cyclase signaling mechanism, the adenylate cyclase-stimulating effect of insulin would not change under these conditions. Hence, in our case, the discovered inhibitory effect of calphostin C will be also extended on the phorbol-insensitive PKC isoforms. Of the latter, the most probable candidate for positive regulation of adenylate cyclase signaling system by insulin may be PKC

ζ, which is activated by PtdIns-3,4,5-P_3, the product of PI3K-catalyzed reactions triggered by insulin [8]. Our data on the inhibition of insulin signal transduction via adenylate cyclase signaling system by wortmannin strongly confirm the above assumption. It should be also noted that PKC ζ was found in the majority of tissues [58].

Based on the ability of PKC (PKC ζ in particular) to phosphorylate both the G_s protein and adenylate cyclase [53, 55], this enzyme may be placed upstream of G_s protein in the adenylate cyclase signaling cascade. The assumption that growth factors, including insulin, can regulate adenylate cyclase activity (type V adenylate cyclase, in particular) via PKC ζ was also made earlier by other authors [53].

In conclusion, we suggest that the adenylate cyclase signaling mechanism of insulin action first discovered by us earlier, in addition to the previously identified functional components (the receptor tyrosine kinase, G_s protein, and adenylate cyclase [1-5, 39, 40]), also comprises the G_i protein, PI3K, and atypical PKC isoforms (PKC ζ).

The data obtained in this study and those published in literature are suggestive of the following cascade of insulin signal transduction to adenylate cyclase.

First stage. Insulin-induced activation of the receptor tyrosine kinase leads to activation of heterotrimeric G_i protein due to dissociation of the latter to α_i subunit and βγ dimer. There are both theoretical and experimental evidences that insulin receptor directly interacts with $G_{i/o}$ proteins [6, 59, 60].

Second stage. The released βγ dimer activates PI3K (the latter catalyzes phosphorylation of phosphoinositides in position D3, yielding 3-phosphoinositides [53]).

Third stage. The product of PI3K-catalyzed reaction PtdIns-3,4,5-P_3 (and, possibly, PtdIns-3,4-P_2) activates atypical PKC isoforms (including PKC ζ) [8, 9].

Fourth stage. PKC ζ phosphorylates and thereby stimulates either G_s protein or directly adenylate cyclase (type V or type II adenylate cyclase, in particular) [55, 56].

This cascade of signaling events results in stimulation of adenylate cyclase activity and enhancement of cAMP synthesis. There are strong reasons to assume that this adenylate cyclase signaling mechanism, first, mediates the effect of not only insulin, but also other peptides of the insulin superfamily [1-5]. Second, according to our hypothesis [39, 40], this mechanism ensures mitogenic rather than metabolic effect of these peptides. The data reported in literature on the key role of PI3K and PKC ζ in mitogenic signal transduction [61, 62] also corroborate this hypothesis.

That fact that we did not find marked differences in the regulatory effect of insulin on adenylate cyclase signaling system in muscles of phylogenetically distant animals, such as mollusks (invertebrates) and rats (vertebrates), indicates that adenylate cyclase signaling mechanism of insulin action is evolutionarily conserved. This is probably due to conservation of the primary structure, as well as structural and functional organization, of the protein components of the insulin-regulated adenylate cyclase system in vertebrates and invertebrates [6, 63-65]. In view of this, it should be noted that mollusks (*A. cygnea*) contain an insulin-like substance that is classified with the insulin superfamily peptides. This substance exerts some regulatory effects similar to those of insulin of vertebrates [66] (in particular, activating effect on mollusk muscle adenylate cyclase) [1, 3].

Thus, we for the first time showed that insulin mediates its stimulating effect on adenylate cyclase via a signaling mechanism that involves the following components: receptor tyrosine kinase → G_i protein (βγ) → PI3K → protein kinase C (ζ?) → G_s protein → adenylate cyclase.

This study was supported by the Russian Foundation for Basic Research (grant No. 00-04-49465).

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