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On the Tyrosine Kinase Mechanism of the Novel Effect of Insulin and Insulinlike Growth Factor I

STIMULATION OF THE ADENYLYL CYCLASE SYSTEM IN MUSCLE TISSUES

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ABSTRACT. For the first time, insulinlike growth factor I (IGF-I), like insulin (Pertseva *et al.*, *Comp Biochem Physiol* **112:** 689–695, 1995), was shown to exercise a GTP-dependent stimulating action on adenylyl cyclase (AC; EC 4.6.1.1.) activity in the muscle tissues (membrane fraction) of mammal (rat) and mollusc (*Anodonta cygnea*). By studying the mechanism of the effect of peptides with selective inhibitors of tyrosine kinase activity, tyrphostin 47 (RG50864, 3,4-dihydroxy-α-cyanothiocinnamamide) and genistein (4,5,7-trihydroxyisoflavone), it was found that receptor tyrosine kinase is involved in this action. The data obtained suggest that the stimulating effect of insulin and IGF-I is produced via the following signalling system: receptor tyrosine kinase → stimulatory G-protein → AC. Thus, the existence of a novel signalling pathway of transduction of signals generated by insulin and related peptides was hypothesised. *Copyright © 1996 by Elsevier Science Inc.* BIOCHEM PHARMACOL 52;12:1867–1874, 1996.

KEY WORDS. insulin; insulinlike growth factor I; stimulation of adenylyl cyclase; tyrosine kinase mechanism

Despite significant progress in the study of the molecular mechanisms of action of insulin and related peptides and the spectrum of their regulatory effects, the phenomenon has not been completely understood. This lack of understanding was made clear by the findings of our previous study, which showed the existence of a GTP-dependent stimulating effect, unknown earlier, of insulin on AC† activity in the muscle tissues of vertebrates (rat) and invertebrates (mollusc *Anodonta cygnea*) [1]. ILP isolated from *A. cygnea* [2] and mammalian EGF have a similar effect [1]. The regulatory influence exercised on the target cells by relaxin and bombyxin, the other peptides of the insulin superfamily, is also produced via activation of AC [3, 4]. Although many attempts have been made to describe the role of the cAMP system in the mechanism of action of insulin and related peptides, the role remains obscure.

It is currently believed that the main mechanism of action of insulin and related peptides, including IGF-I and other growth factors, e.g. EGF, is a protein tyrosine phosphorylation cascade catalysed by protein tyrosine kinase of the peptide receptors [5–7]. However, activation of the insulin receptor tyrosine kinase has been shown to be of prime importance for a number of, but not all, biological effects induced by insulin.

To investigate the stimulating effect of insulin and related peptides on the AC signalling system further, particular attention was paid to two tasks. First, to enlarge the list of peptides of the insulin superfamily under study, the ability of IGF-I, which possesses a great similarity to insulin in its primary structure and in the design of receptor tyrosine kinase [8, 9], to activate AC was studied. Second, an attempt was made to determine whether the tyrosine kinase mechanism participates in the realization of ACSE of insulin and IGF-I, which was done by studying the influence of selective inhibitors of tyrosine kinases (tyrphostin 47 and genistein) [10] on this effect. In this series of experiments, the effect of insulin and IGF-I was compared with that of EGF, which also possesses receptor tyrosine kinase and exercises a GTP-dependent ACSE in the tissues of vertebrates [11–13]. Because these inhibitors are capable of disturbing the tyrosine kinase function of receptor of insulin and of growth factors (see [10]), they are widely used as tools to study signal transduction processes in which protein tyrosine kinases are implicated.

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† *Abbreviations:* AC, adenylyl cyclase (EC 4.6.1.1.); IGF-I, insulinlike growth factor I; EGF, epidermal growth factor; ILP, insulinlike peptide isolated from mollusc *Anodonta cygnea*; ACSE, adenylyl cyclase stimulating effect of peptides; Gpp[NH]p, 5′-guanylylimidodiphosphate; G-protein, GTP-binding protein; G_s, G-protein of stimulatory type.

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MATERIALS AND METHODS

Chemicals and Radiochemicals

Creatine phosphate, creatine phosphokinase, GTP, Gpp[NH]p, Tris HCl, alumina for column chromatography ATP, cAMP and imidazole were obtained from Sigma (St. Louis, MO, USA). [α-^{32}P]ATP (4 Ci/mmol) was obtained from Isotop (St. Petersburg, Russia).

Peptides

Mammalian insulin (24 IU) was obtained from Lilly (Indianapolis, IN, USA), recombinant human IGF-I was obtained from Amersham (Buckinghamshire, UK), and EGF was isolated from mouse submaxillary glands by the method described in [1, 4].

Protein Tyrosine Kinase Inhibitors

Tyrphostin 47 (3,4-dihydroxy-α-cyanothiocinnamamide, RG50864; Sigma) is from the family of highly selective blockers (benzylidene derivative) of receptor tyrosine kinase, which are synthetic analogues of the microbe inhibitor of tyrosine kinase erbstatin [10]. Irrespective of the mode of action, tyrphostins are, as a rule, 100–10,000 times more potent in inhibiting protein tyrosine kinases than are protein serine/threonine kinases (protein kinase C, A, Ca^{2+} calmodulin dependent, etc.). Genistein is a natural bioflavonoid (4,5,7-trihydroxyisoflavone, Sigma) capable of producing a specific inhibitory effect on tyrosine kinase activity while having almost no effect on the activity of protein kinase of the serine/threonine type [15].

The effects of peptides and blockers were studied *in vitro* by adding these agents to the sample for determination of AC activity. The duration of action of peptides was limited to 2.5 min, time enough for the effect to reach its peak [1]. The blockers were preincubated with a sample for 15 min, followed by the addition of peptides (duration of action, 2.5 min) at a concentration giving a maximal ACSE [1]. Samples treated in the same manner with solvents for peptides and blockers were used as a control.

Membrane Preparation

The investigation was performed on sarcolemma membrane fractions isolated from the foot (smooth) muscle of freshwater bivalve mollusc, *A. cygnea*, and the skeletal muscle of rat, *Rattus norvegicus* (for each fraction, 25–30 molluscs and 4–6 rats were used), following the method of Kidwai *et al.* [16].

AC Assay

AC activity was determined by the method of Salomon *et al.* [17] with some modifications. The reaction mixture (final volume = 50 μL) contained 50 mM Tris (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 creatine phosphokinase and 15–20 mg of membrane protein. Incubation was carried out at 37°C (rat) and 30°C (molluscs) for 2.5 min. cAMP was determined according to the method of White [18] by using alumina in column chromatography. All experiments were performed in triplicate at least three times.

Protein Assay

The protein content was determined according to the method of Lowry *et al.* [19] by using bovine serum albumin as a standard compound.

Statistical Analysis

The values are presented as the means ± SEM. Student's paired *t*-test was used to test the differences between experimental groups (Fig. 2 and Table 2). Differences with a p value of less than 0.05 were considered significant.

RESULTS AND DISCUSSION

IGF-I Influence on AC Activity in Mollusc and Rat Muscles

Figure 1 shows that IGF-I (10^{-11}–10^{-6} M) exercised a stimulating effect on AC activity in the muscles of mollusc *A. cygnea* (Fig. 1A) and rat (Fig. 1B). The concentration dependence of the effect, however, was different. The maximal effect was reached at the IGF-I concentration of 10^{-8} M and 10^{-9} M in the mollusc (Fig. 1A) and rat (Fig. 1B) muscles, respectively. Moreover, the effect was higher in rat (+95%) than in mollusc (+54%). The sensitivity of tissue of the mammal to mammalian IGF-I is apparently higher than that of tissue of mollusc. A similar regularity was observed in our previous study [1] with respect to ACSE of mammalian insulin in muscle tissues of rat and *A. cygnea*. Thus, species specificity of the peptide effects was revealed.

The concentration-dependent curve of the IGF-I effect deviates from a typical form (Fig. 1). We observed a similar bell-shaped curve in the case of the AC effect of insulin and mollusc insulinlike peptide [1]. Proceeding from the kinetic model developed by Kühl [20] to account for the phenomenon of high-dose inhibition in the receptor/agonist system, the descending limb of the curves can be ascribed either to receptor desensitization arising at supramaximal concentration of agonist or to the existence of two types of receptor, which high and low affinity, coupled with stimulatory and inhibitory signalling pathways of hormone action, respectively.

The ACSE of IGF-I was shown to be GTP dependent (Fig. 2). The nonhydrolysable analogue of GTP, Gpp[NH]p (10^{-5} M), had a potentiating influence on the peptide activation of AC (an increase over the additive effect of both agents in *A. cygnea* and rat muscles by 135% and 50%, respectively). The effect of guanine nucleotide suggests the involvement of a G_s in the realization of IGF-I

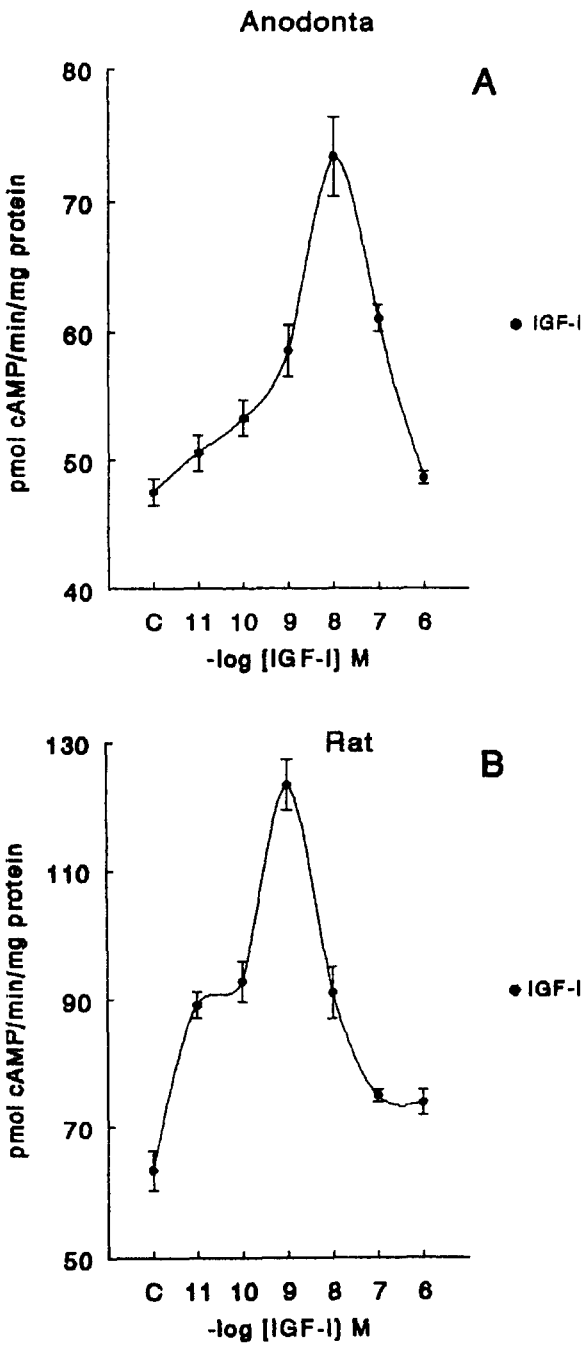

FIG. 1. Concentration dependence of the stimulating action of IGF-I on AC activity in *A. cygnea* (A) and rat (B) muscles. (A, B) Vertical axis, AC activity in pmol cAMP/min/mg protein; horizontal axis, (-)log of IGF-I concentration (M); C, control. Each point represents the mean of 3–4 values. The plotted data are the result of a typical experiment.

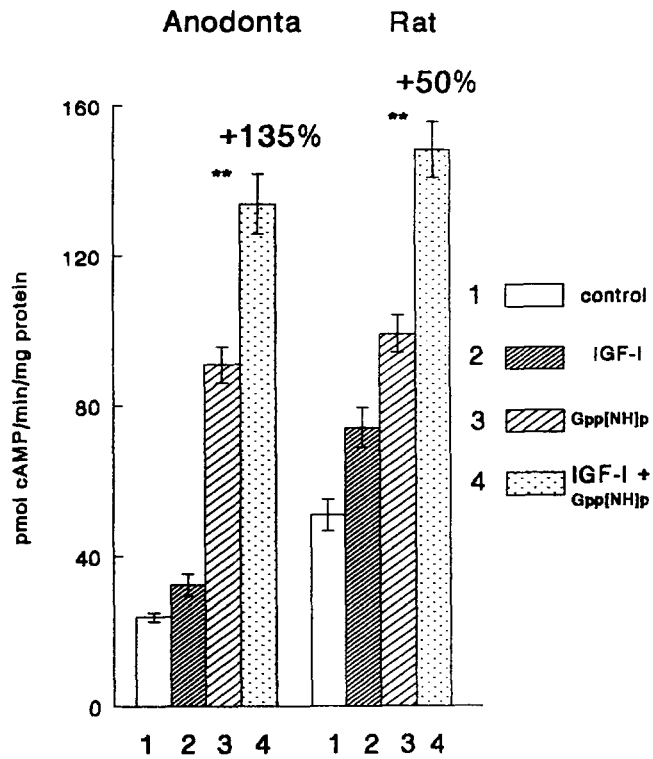

FIG. 2. Potentiating influence of Gpp[NH]p on the AC stimulating effect of IGF-I in *A. cygnea* and rat muscles. Vertical axis, AC activity in pmol cAMP/min/mg protein; horizontal axis, 1 = control, 2 = IGF-I (10^{-8} M for *A. cygnea* and 10^{-9} M for rat), 3 = Gpp[NH]p (10^{-5} M), 4 = IGF-I + Gpp[NH]p. Figures above bar 4 indicate the potentiating effect (% increment over the additive effect of IGF-I and Gpp[NH]p, acting separately). The data were expressed as the means ± SEM (n = 4). The paired *t*-test was used for the potentiating effect of Gpp[NH]p; a comparison was made between bars 2 and 3 (arithmetical sum of insulin and Gpp[NH]p effects) and bar 4; $^{**}p < 0.01$.

ACSE. Such a G-protein, a substrate of cholera toxin (45–50 kDa doublet), was detected in the *A. cygnea* muscles in our earlier work [21]. G_s was also identified in the nervous tissue of the mollusc *Lymnaea stagnalis* [22].

The ability of IGF-I, like that of insulin, to stimulate AC activity GTP dependently provides further evidence in support of a similarity of the mechanism of action of these two peptides.

The Influence of Receptor Tyrosine Kinase Blockers on Transduction of Insulin and EGF Signals via the AC System

Tyrphostins and genistein have been used extensively in an attempt to establish whether tyrosine phosphorylation plays a role in the transmission of different biochemical signals, e.g. those generated by insulin and EGF receptor kinases (see [10]).

MOLLUSC MUSCLES. Due to the influence of different concentrations of tyrphostin 47 (1.25–10 μM) on the ACSE of insulin (10^{-8} M) in the muscle membrane fraction of *A. cygnea* (Fig. 3A), a dose-dependent inhibition of the effect was observed. Suppression of the effect reached its peak (a 70–80% decrease) at a tyrphostin concentration of 5.0 μM and higher.

The ACSE of EGF (10^{-9} M) (Fig. 3A) was also blocked

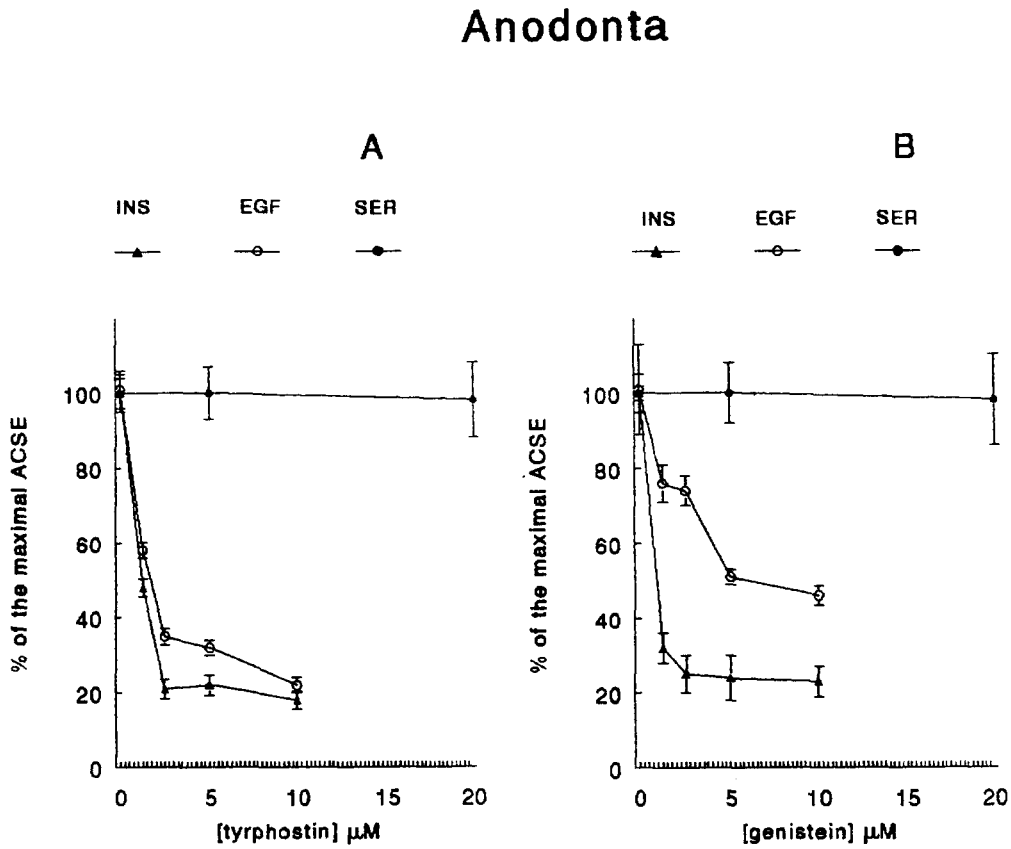

FIG. 3. Effect of tyrphostin 47 (A) and genistein (B) on the ACSE of insulin, EGF and serotonin in *A. cygnea* muscles. (A, B) Vertical axis, % maximal AC stimulating effect (taken as 100%) of insulin (10^{-8} M), EGF (10^{-9} M) and serotonin (10^{-5} M); horizontal axis, tyrphostin (A) and genistein (B) concentration (μM). Each point represents the mean of 3–4 experiments, each performed in triplicate.

by tyrphostin 47. The concentration dependence coincides with that observed in the insulin effect. A comparison of IC_{50} values of tyrphostin inhibitory action shows that for the EGF ACSE this value was somewhat higher than that for the insulin ACSE (IC_{50} = 1.22 μM and 1.03 μM, respectively) (Table 1).

To check the specificity of tyrphostin 47 action on the tyrosine kinase activity of insulin receptor in the mollusc muscles, we determined under the same experimental conditions the serotonin-stimulated AC activity mediated through the receptor of nontyrosine kinase type coupled with G_s [23] and found it practically unchanged (Fig. 3A).

TABLE 1. IC_{50} values for the blocking action of tyrphostin 47 and genistein on the ACSE of insulin, IGF-I and EGF in *A. cygnea* and rat muscles

Additions	IC_{50} of Tyrphostin (μM)		IC_{50} of Genistein (μM)	
	A. cygnea	Rat	*A. cygnea*	Rat
Insulin 10^{-8} M	1.03	0.78	0.75	1.10
EGF 10^{-9} M	1.22	—	1.88	—
10^{-10} M	—	2.75	—	1.70
IGF-I 10^{-9} M	—	0.53	—	1.20

In the following series of experiments, another inhibitor of receptor tyrosine kinase, genistein, was used. At a concentration of 1.25–10 μM (Fig. 3B), it inhibited the ACSE of both insulin and EGF; at a concentration of 5.0 μM and higher, inhibition reached 50–75%. The IC_{50} value of genistein action was lower for insulin than for the EGF effect (IC_{50} = 0.75 and 1.88 μM, respectively; Table 1). Genistein had no blocking action on serotonin-stimulated AC activity (Fig. 3B).

When the influence of tyrphostin 47 and genistein on the ACSE of insulin and EGF taken at different concentrations (10^{-11}–10^{-6} M and 10^{-11}–10^{-7} M, respectively) was investigated, the effect was always inhibited (data not shown).

RAT SKELETAL MUSCLES. Tyrphostin 47 (0.5–10 μM) in the rat skeletal muscles exercised an inhibitory influence on the ACSE of both insulin (10^{-8} M) and EGF (10^{-10} M) (Fig. 4A). The concentration dependence of the blocking effect of tyrphostin was observed within the concentration limit of 0.5–5 μM. At a tyrphostin concentration of 5.0 μM and higher, the effect of insulin and EGF was blocked to the greatest degree (approximately 50%). The IC_{50} value was 0.78 μM for insulin and 2.75 μM for EGF (Table 1). Unlike the ACSE of insulin and EGF, tyrphostin (5 and 20

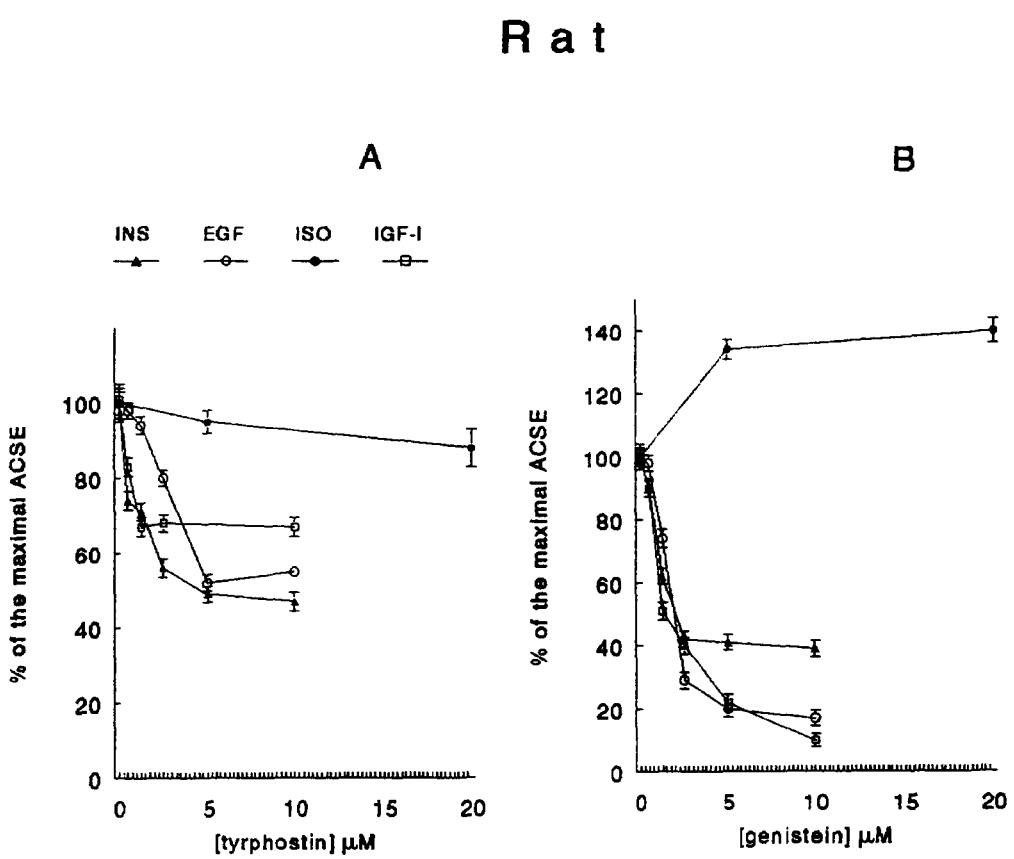

FIG. 4. Effect of tyrphostin 47 (A) and genistein (B) on the ACSE of insulin (10^{-8} M), IGF-I (10^{-9} M), EGF (10^{-10} M) and isoproterenol (10^{-5} M) in rat muscles. All designations are the same as in Fig. 3, except that isoproterenol rather than serotonin was used.

μM) scarcely inhibited the effect of isoproterenol (Fig. 4A). In a study [24] carried out on muscle membranes of the rat heart, tyrphostins also blocked the EGF ACSE but did not change the effect of isoproterenol.

The other tyrosine kinase blocker, genistein, at a concentration of 0.5–10 μM, suppressed (maximally by 60–80%) the stimulating effect of insulin and EGF on AC activity and did not block the isoproterenol effect (Fig. 4B). The IC_{50} values of genistein action were 1.10 μM for insulin and 1.70 μM for the EGF (Table 1). Nair and Patel [24] showed that genistein inhibited the ACSE of both EGF and isoproterenol in rat cardiac membranes. This result can be ascribed to the fact that Nair and Patel used genistein at a concentration (400 μM) 40 and more times higher than in our study, which is the reason their conclusion concerning the failure of genistein as an inhibitor of receptor tyrosine kinase being due to its nonselectivity is not likely to be correct. Our study showed that a specific concentration-dependent inhibition of ACSE was induced by genistein at a low concentration (0.5–5.0 μM). The ability of genistein and tyrphostin (AG82) to block the contractile effect of vascular agonists [25], considered by the authors to be realized via the tyrosine kinase mechanism, was also observed in the range of 2.0–5.0 μM. Thus, the analogous effect of norepinephrine was not suppressed.

In the rat skeletal muscles, tyrphostin 47 and genistein exercised a blocking influence on the ACSE of insulin and EGF, observed not only at the concentration giving maximal effect (10^{-8} M and 10^{-10} M, respectively) but also at other concentrations of the peptides (insulin: 10^{-10} – 10^{-6} M and EGF: 10^{-11} – 10^{-7} M) (data not shown).

Our findings concerning a blocking action of these inhibitors on ACSE being induced by insulin and EGF are in accord with the data in the literature (see [10]) on the damaging action of these agents on EGF- and insulin-receptor tyrosine kinases and some insulin-induced distal effects.

That tyrphostin 47 and genistein exercise inhibitory action on the receptor level of the signalling system involved in the realization of ACSE is confirmed by our data showing that this was not the case on the distal level, $G_s \rightarrow AC$ (Table 2). In the absence of peptides, the two blockers not only did not inhibit but actually increased basal and Gpp[NH]p-stimulated AC activity in the mollusc and rat muscles. The reason for this is not yet clear. In the rat heart muscles [24], tyrphostins did not decrease the level of basal and Gpp[NH]p-stimulated AC activity, as was true for the present study.

The Influence of Receptor Tyrosine Kinase Blockers on IGF-I-Induced ACSE in Rat Muscles

The IGF-I ACSE similar to that of insulin and EGF was inhibited by tyrphostin 47 in the rat skeletal muscles (Fig.

TABLE 2. Influence of tyrphostin 47 and genistein on the basal and Gpp[NH]p-stimulated AC activity in *A. cygnea* and rat muscles

Additions	AC activity (pmol cAMP/min/mg protein)	
	-Gpp[NH]p	+Gpp[NH]p
A. cygnea		
Control	8.0 ± 0.29	12.7 ± 1.3
Tyrphostin 5 μM	13.3 ± 0.22** (+66)	23.5 ± 0.94** (+74)
Genistein 5 μM	9.8 ± 0.67* (+23)	15.1 ± 1.3 (+52)
Rat		
Control	3.6 ± 0.55	7.7 ± 1.12
Tyrphostin 5 μM	10.3 ± 1.52** (+190)	37.3 ± 2.53** (+384)
Genistein 5 μM	5.0 ± 1.10 (+39)	8.7 ± 1.35 (+12)
Control	3.6 ± 0.55	7.7 ± 1.12
Tyrphostin 10 μM	14.2 ± 1.87* (+297)	20.5 ± 2.14** (+165)
Genistein 10 μM	11.3 ± 1.91* (+219)	26.7 ± 2.64** (+246)

Note: The concentration of Gpp[NH]p is 10^{-5} M. The treatment of the membrane fraction by tyrphostin or genistein was carried out according to the procedures described in Materials and Methods. Numbers within parentheses indicate the effect of inhibitors as percentage of control. *$p < 0.05$; **$p < 0.01$, paired *t*-test.

4A). Concentration dependence was observed in the range of 0.5–1.25 μM. A visible blocking effect (30%) was already attained at a tyrphostin concentration of 1.25 μM, the IC_{50} value for tyrphostin being 0.53 μM (Table 1).

Genistein also exercised a dose-dependent inhibitory action on the ACSE of IGF-I (0.5–5.0 μM), with a maximal inhibition of 80–90% (Fig. 4B). The IC_{50} value for genistein was 1.20 μM (Table 1).

These data on the influence of the specific blockers (tyrphostin 47 and genistein) of receptor tyrosine kinase on the ACSE of insulin, IGF-I and EGF yielded a similar picture in the muscle tissues of rat and mollusc. The inhibitors at micromolar concentrations (0.5–10 μM) distinctly blocked the effect of peptides in both tissues. The inhibitory influence of the blockers was specific for receptor tyrosine kinase because it was lacking in the case of stimulating action of biogenic amines (serotonin, isoproterenol), which is realised through the receptors possessing no tyrosine kinase activity. A comparison of IC_{50} values (Table 1) shows that the sensitivity of the systems involved in insulin and IGF-I ACSE to tyrosine kinase inhibitors was somewhat higher than that of EGF in the rat and mollusc muscles. These data differ from those of other authors [10, 26, 27] who showed a significantly higher sensitivity to tyrphostins: RG50864 (tyrphostin 47 used in our experiments), RG50810 and genistein of receptor tyrosine kinase of EGF compared with that of insulin. This discrepancy may be due to some species or tissue differences in the receptor tyrosine kinases. It follows from the present work that tyrphostin 47 (and possibly genistein) seems to be a more specific blocker of receptor tyrosine kinase of insulin and IGF-I than of EGF. This selective blocker can exert action at a concentration one order lower than that of the tyrphostin analogues used in [26].

As far as the target of the blocking action of tyrphostin 47 and genistein within the signalling pathway of peptide ACSE is concerned according to the available data, these inhibitors of receptor tyrosine kinase do not affect the process of ligand-receptor interaction (EGF, insulin) [10, 26]. Consistent with the literature [24, 25], our data show that the blockers exercised no inhibitory influence on the postreceptor stages leading to the activation of AC (via G_s) induced by agents (e.g. biogenic amines) whose receptors lack tyrosine kinase. These blockers did not inhibit the basal and Gpp[NH]p-stimulated AC activity. Thus, the process of signal transduction generated by insulin, IGF-I and EGF was blocked at the level of receptor tyrosine kinase. This conclusion is supported by the results of a study that showed inhibition by tyrphostins of the receptor tyrosine kinase of a number of growth factors and of insulin and a decrease in tyrosine phosphorylation of some of tyrosine kinase protein substrates [10].

The mechanism of the blocking action of tyrphostins and genistein has not been studied in detail. Levitzki [10] showed that different tyrphostins have different mechanisms of inhibitory action based on competition with tyrosine kinase for protein substrates of the enzyme, for ATP, or both. Genistein has been shown to be an ATP competitor selective in inhibiting tyrosine kinases [10]. In the present work, no significant difference was found in the potency of the inhibitory action of tyrphostin 47 and genistein on the ACSE of peptides in mollusc and rat muscles.

The results of our study allow the following conclusions.

(1) IGF-I, insulin and ILP of mollusc *A. cygnea*, the members of the insulin superfamily [25, 28], have ACSE in the muscle tissues of mollusc and rat, phylogenetically remote animals. The potentiating influence of guanine nucleotides on the ACSE of insulin, ILP and IGF-I ([1] and the present study) provides evidence for the participation of G_s in the realization of ACSE. This finding is the first to support the results of Okamoto *et al.* [29] concerning the existence in the primary structure of an insulin receptor cytoplasmic region of a sequence capable of specific binding and activating G_s. Until now there were no data to show that insulin receptor in the cell can functionally interact with G_s followed by stimulation of AC. Hence, there are grounds to suggest the existence of such a domain in the receptors of other members of the insulin superfamily, e.g. IGF-I. A similar domain was found in the EGF receptor [30], which is in agreement with the ability of EGF to activate AC via receptor tyrosine kinase and G_s [11, 24].

(2) The ACSE of insulin, IGF-I and EGF is produced by the tyrosine kinase mechanism in the muscle tissues of rat and mollusc, as indicated by the concentration-dependent

inhibitory action on the peptide ACSE of selective blockers of receptor tyrosine kinases (tyrphostin 47 and genistein). The data obtained in the study of mollusc (*A. cygnea*) muscles suggest the presence in the tissue of a receptor of the tyrosine kinase type that can be implicated *in vivo* in the ACSE of ILP of *A. cygnea* [1]. The gene encoding the receptor of such a type was detected in the mollusc *Lymnaea stagnalis* [31].

(3) Our data and those from the literature [3, 4] suggest that the activating action on the AC system is characteristic of a number of peptides of the insulin superfamily and some growth factors (e.g. EGF) united by a common feature: the presence of receptor tyrosine kinase. The mechanism of the effect of peptides may involve the following signalling system: receptor tyrosine kinase $\rightarrow G_s \rightarrow$ AC. According to our hypothesis [1], prompted by a new approach to cAMP as a positive regulator of cell proliferation [32], this new signalling system is engaged in a mitogenic rather than a metabolic action of peptides of the insulin superfamily and some growth factors.

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References

1. Pertseva MN, Plesneva SA, Shpakov OA, Rusakov Y and Kuznetsova LA, Involvement of the adenylyl cyclase signalling system in the action of insulin and mollusc insulin-like peptide. *Comp Biochem Physiol* **112:** 689–695, 1995.
2. Rusakov Y, Bondareva VM, Karasev VS, Leibush BN, Barkan RS and Pertseva MN, Some biochemical characteristics of an insulin-like substance of the bivalve mollusc *Anodonta cygnea*. *Biokhimiya* **56:** 718–726, 1991 (In Russian).
3. Bryant-Geenwood GD and Schwabe C, Human relaxins: Chemistry and biology. *Endocrinol Rev* **15:** 5–26, 1994.
4. Filippovich Y and Kutusova NM, The hormone regulation of metabolism in insects. In: *The General Results of Science And Technology, Biological Chemistry*, vol. 21 (Ed. Mikhailov AI), p. 226. Viniti, Moscow, 1985 (In Russian).
5. White MF and Kahn CR, The insulin signalling system. *J Biol Chem* **269:** 1–4, 1994.
6. Schlessinger J and Ullrich A, Growth factor signalling by receptor tyrosine kinases. *Neuron* **9:** 383–391, 1992.
7. Hernandez-Sotomayor SMT and Carpenter G, Epidermal growth factor receptor: Elements of intracellular communication. *Membr Biol* **128:** 81–89, 1992.
8. Humbel RE, Insulin-like growth factor I and II. *Eur J Biochem* **190:** 445–462, 1990.
9. Gonborg M, Wulff BS, Rasmussen JS, Kjeldsen T and Gammeltoft S, Structure–function relationship of the insulin-like growth factor I receptor tyrosine kinase. *J Biol Chem* **268:** 23435–23440, 1993.
10. Levitzki A, Tyrphostins: Tyrosine kinase blockers as novel antiproliferative agents and dissectors of signal transduction. *FASEB J* **6:** 3275–3282, 1992.
11. Nair BG, Parikh B, Milligan G and Patel TB, G_s-α mediates epidermal growth factor-elicited stimulation of rat cardiac adenylate cyclase. *J Biol Chem* **265:** 21317–21332, 1990.
12. Budnik LT and Mukhopadhyay AK, Epidermal growth factor, a modulator of luteal adenylate cyclase. Characterization of epidermal growth factor receptor and its interaction with adenylate cyclase in bovine luteal cell membrane. *J Biol Chem* **266:** 13908–13913, 1991.
13. Nakagawa Y, Gammichia J, Parushotham KR, Schneyer CA and Humphreys-Beher MG, Epidermal growth factor activation of rat parotid gland adenylate cyclase and mediation by a GTP-binding regulatory protein. *Biochem Pharmacol* **42:** 2333–2340, 1991.
14. Nikolsky NN, Sorkin AD and Sorokin AB, *Epidermal Growth Factor*. Nauka, Leningrad, 1987 (in Russian).
15. Akijama T, Ishida J, Nakagawa S, Ogawa H, Watanabe S, Ito N, Shibuya M and Fukami Y, Genistein, a specific inhibitor of tyrosine kinase specific protein kinases. *J Biol Chem* **262:** 5592–5597, 1987.
16. Kidwai AM, Radcliffe MA, Lee EY and Daniel EE, Isolation and properties of skeletal muscle membrane. *Biochim Biophys Acta* **298:** 593–607, 1973.
17. Salomon Y, Londos C and Rodbell M, A highly sensitive adenylate cyclase assay. *Anal Biochem* **58:** 541–548, 1974.
18. White AA, Separation and purification of cyclic nucleotides by aluminia column chromatography. *Methods Enzymol* **380:** 41–46, 1974.
19. Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
20. Kühl PW, Excess-substrate inhibition in enzymology and high-dose inhibition in pharmacology: A reinterpretation. *Biochem J* **298:** 171–180, 1994.
21. Pertseva MN, Kuznetsova LA, Plesneva SA, Grishin AV and Panchenko MP, β-Agonist inhibitory-guanine-nucleotide-binding regulatory protein coupling to adenylate cyclase in mollusc *Anodonta cygnea* foot muscle sarcolemma. *Eur J Biochem* **210:** 279–286, 1992.
22. Knol JC, Weidemann W, Planta RJ, Vreugdenme E and Heerikhuizen H, Molecular cloning of G protein alpha-subunits from the central nervous system of the mollusc *Lymnaea stagnalis*. *FEBS Lett* **314:** 215–219, 1992.
23. Kuznetsova LA, Plesneva SA, Derkach KV and Pertseva MN, Effects of biogenic amines and glucagon on the adenylate cyclase system in molluscan, holothurian and ascidian muscle membranes. *Comp Biochem Physiol* **111C:** 293–301, 1995.
24. Nair BG and Patel TB, Regulation of cardiac adenylyl cyclase by epidermal growth factor (EGF). Role of EGF receptor protein tyrosine kinase activity. *Biochem Pharmacol* **46:** 1239–1245, 1993.
25. Laniyonu AA, Saifeddine M, Yang S-G and Hollenberg MD, Tyrosine kinase inhibitors and the contractile action of G-protein-linked vascular agonists. *Can J Physiol Pharmacol* **72:** 1075–1085, 1994.
26. Lyall RM, Zilberstein A, Gazit A, Gilon C, Levitzki A and Schlessinger J, Tyrphostins inhibit epidermal growth factor (EGF)-receptor tyrosine kinase activity in living cells and EGF-stimulated cell proliferation. *J Biol Chem* **264:** 14503–14509, 1989.
27. Linassier C, Pierre M, Le Peco J-B and Pierre J, Mechanisms of action in NIH-3T3 cells of genistein, an inhibitor of EGF receptor tyrosine kinase activity. *Biochem Pharmacol* **39:** 187–193, 1990.
28. Plesneva SA, Shpakov AO, Rusakov Y, Kuznetsova LA, Pertseva MN, The involvement of adenylate cyclase signalling system in the action of insulin, epidermal growth factor and insulin-like peptide of mollusc. In: *Proceedings of the 17th Conference of European Comparative Endocrinologists, Universiteit,*

Cordoba, Spain, 5–10 September 1994 (Ed. Roubos EW). University Press, Cordoba, 1994.
29. Okamoto T, Murayama Y, Hayaschi Y, Ogata E and Nishimoto I, GTP-binding protein-activator sequences in the insulin receptor. *FEBS Lett* **334:** 143–148, 1993.
30. Sun H, Seyer JM and Patel TB, A region in the cytosolic domain of the epidermal growth factor receptor antithetically regulates the stimulatory and inhibitory guanine nucleotide-binding regulatory proteins of adenylyl cyclase. *Proc Natl Acad Sci USA* **92:** 2229–2233, 1995.
31. Roovers JFA, van Heerikhuizen H, Ceraerts WPM, Joose J and Planta RJ, The isolation of genes encoding the receptor(s) for molluscan insulin-related peptides (MIPs) from the freshwater snail *Lymnaea stagnalis*. In: *Proceedings of the Symposium on Molluscan Neurobiology, Vrije Universiteit, Amsterdam, The Netherlands, 20–24 August 1990* (Ed. Roubos EW), p. 121. University Press, Amsterdam, 1990.
32. Dumont JE, Jauniaux J-C and Roger PP, The cyclic AMP-mediated stimulation of cell proliferation. *TIBS* **14:** 67–71, 1989.