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LOW INSULIN CONCENTRATIONS STIMULATE IN VITRO THE SOLUBLE GUANYLATE CYCLASE ACTIVITY OF RAT LIVER

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Summary The soluble guanylate cyclase activity of rat liver appears to be stimulated in VITRO by insulin at pMolar concentrations, while proinsulin, denaturated insulin or desoctapeptide insulin, are not able to stimulate the studied enzymic activity. Corresponding concentrations of other peptide hormones such as corticotropin (ACTH) or glucagon , either in the absence or in the presence of bacitracin ,do not show any effect on the investigated enzymic system.

Insulin stimulation of the soluble guanylate cyclase is characterized by a significant increase in the Vmax together with a decrease of the appearent Km.

Insulin at low concentrations doesn't affect the cyclic GMP hydrolyzing activity;conversely higher concentrations of the hormone, while exerting a less marked effect on the guanylate cyclase activity, inhibit the cyclic GMP hydrolyzing activity.

The intracellular levels of cyclic AMP and cyclic GMP are affected by insulin in a coordinate and reciprocal way; while the basal or "stimulated" cyclic AMP level is lowered (1-6) , the cyclic GMP one is increased (7,8).The enzymatic mechanisms underlying the effect on the cyclic AMP level have been extensively investigated: they inhibit both the basal and the stimulated adenylate cyclase (9-14) and stimulate the hydrolyzing activities on the cyclic AMP (15-20).

As far as the cyclic GMP metabolism is concerned , the soluble guanylate cyclase activity has been reported to be activated when extracted from the cells incubated in the presence of rather high, unphysiological insulin concentrations(21).

Our study refers about the in VITRO action of low physiological insulin concentrations, on the soluble guanylate cyclase activity and was suggested by the established evidence that insulin , after a primary action on the membrane level (22) , moves laterally through the membrane plane as a hormone - receptor complex (23) and is actively transported inside the cell (24,25) , where its action is still largely unknown (26).

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Materials and Methods

Materials: GTP, cyclic GMP, creatine phosphate , creatine-phospho-kinase were purchased from Boehringer (Mannheim, WG). Corticotropin (ACTH),3-iso-butil- methil-xanthine (IBMX),sodium azide (NaN_3) from Sigma (St. Louis ,MO, USA); imidazole from BDH (England); neutral alumina from Merck (Darmstadt ,W.G.) ; α^{32}P GTP , ^{3}H cyclic GMP were from New England Nuclear (Boston, MA, USA). Native pork insulin and glucagon were a generous gift of Dr.I.Parikh of the Wellcome Research Laboratories (NC, USA). Proinsulin and desoctapeptide insulin were a generous gift of Dr. R. Carroll and D.F.Steiner of the University of Chicago, USA. All the other reagents were of the highest available purity grade

Preparation of the enzymic source::the livers were removed from Sprague-Dawley rats (150-200 gr.) killed by decapitation,perfused through the portal vein with 30 ml of ice cold 50 mM Tris-HCl (pH 8.0) buffer containing 0.25 M sucrose. All the subsequent procedures were performed at 4°C. The livers were homogenized in 4 volumes of buffer in a Potter apparatus equipped with Teflon glass homogenizer for 30 sec in two following strokes. The homogenate was centrifuged at 800 x g for 15 min. The supernatant was recentrifuged at 105,000 x g for 60 min. The supernatant fraction was utilized as enzymic source.

Assay of guanylate cyclase activity::the incubation medium contained in a final volume of 0.1 ml 50 mM Tris-HCl buffer pH 7.6,4 mM $MnCl_2$,1 mM GTP,0.5 mM IBMX and 1.5 μC α^{32}P GTP (S.A. 10-50 Ci/mmol), creatine kinase (1 mg/ml),30 mM creatine phosphate.Proinsulin,insulin,desoctapeptide insulin and glucagon first dissolved respectively in 0.2% sodium bicarbonate and 6 M guanidine were properly diluted in 50 mM Tris-HCl buffer pH 7.6 before the addition immediately. The reaction was started by adding 80-100 μg of membrane proteins and carried out at 37°C for 10 min, stopped by the addition of 100 ul of 50 mM Tris-HCl buffer pH 7.6,3 mM cyclic GMP, 0.1 mM EDTA and ^{3}H cyclic GMP (S.A. 8-15 Ci/mmol) equivalent to 25-30,000 cpm to determinate the recovery .Each sample was immediately diluted with 0.4 ml of ice cold 0.1 M imidazole HCl buffer pH 7.5 and absorbed on neutral alumina columns (29) (0.5 x 2.5 cm) equilibrated with the same buffer .After the addition of 2.0 ml of the equlibrating buffer , a fraction of 2.5 ml was collected,added to 6.0 ml of Instagel scintillation medium and counted for its radioactivity.From each column the recovery of the cyclic GMP was corresponding to 75-85% of the absorbed cyclic GMP. One series of experiments was carried out in the absence of labelled GTP and the formed cyclic GMP was assayed by a RIA method (New England Nuclear, Boston, MA, USA).

Assay of the cyclic GMP hydrolyzing activity:the cyclic GMP hydrolyzing activity was investigated in an incubation medium containing in a final volume of 0.1 ml :10 mM Tris HCl buffer pH 8.0, 0.2 mM dithiothreitol,1.2 mM $MnCl_2$, 1 μM GTP,creatine kinase (1 mg/ml),30 mM creatine phosphate , 1 μM cyclic GMP , 0.5 mM IBMX and ^{3}H cyclic GMP corresponding to 50,000 cpm,added for the determination of the activity. The reaction was started by the addition of the enzymic source (80-100 μg of proteins) and ,carried out at 37°C for 10 min.,stopped by a 3 min boiling. The formed GMP was further splitted to guanosine and phosphate through digestion by venom snake 5' nucleotidase and the labelled guanosine isolated according to Thompson & Appleman (30).

Results and Discussion

The results reported in Table 1 indicate that the soluble guanylate cyclase activity can be increased by insulin at the concentration of 3×10^{-11} M to an extent corresponding to 70% above the control. They also show that insulin effect

Table I

In VITRO effect of insulin, sodium azide (NaN_3), and various peptidic agents on the rat liver cytoplasmatic guanylate cyclase activity.

Addition	Guanylate cyclase activity pMoles of cyclic GMP/mg protein/min
No	91±6
Insulin 3×10^{-11} M	163±11
Insulin 3×10^{-10} M	96±5
Proinsulin 5×10^{-11} M	101±7
Proinsulin 5×10^{-10} M	101±4
Proinsulin 1×10^{-9} M	109±8
NaN_3 1 mM	338±20
NaN_3 10 mM	374±22
NaN_3 1 mM + Insulin 3×10^{-11} M	532±35
NaN_3 10 mM + Insulin 3×10^{-11} M	576±39
Desoctapeptide insulin 3×10^{-11} M	92±4
Denaturated insulin 3×10^{-11} M	91±6
Corticotropin 10^{-8} M	90±7
Bacitracin 5 mg/ml	93±5
Bacitracin 5 mg/ml + Insulin 3×10^{-11} M	170±11
Glucagon 10^{-8} M	94±6
Bacitracin 5 mg/ml + Glucagon 10^{-8} M	93±3

The isolation of the cyclic GMP was obtained through a slight modification of the cromatography procedure on neutral alumina columns described by Siegel et al (29). For other experimental conditions see Methods.
The results are means ±S.D. of three experiments; each one was carried out in triplicate.

is not mimicked by other peptidic structures , related (proinsulin , desoctapeptide insulin and denaturated insulin) or not (glucagon ,corticotropin and bacitracin), to the insulin one. Since bacitracin has been shown to be a substrate for proteolytic enzymes and therefore to protect glucagon from proteolytic degradation (31) , the antibiotic was tested in association with insulin and glucagon; the bacitracin protection from proteolytic degradation neither made glucagon able to display a stimulating effect nor was able to enforce the insulin action. The stimulating effect on the investigated enzymic activity is additive to submaximally or maximally stimulating NaN_3 concentrations. The last results suggest that the insulin effects are mediated by a different indipendent molecolar mechanisms and therefore they shouldn't require oxidative processes.

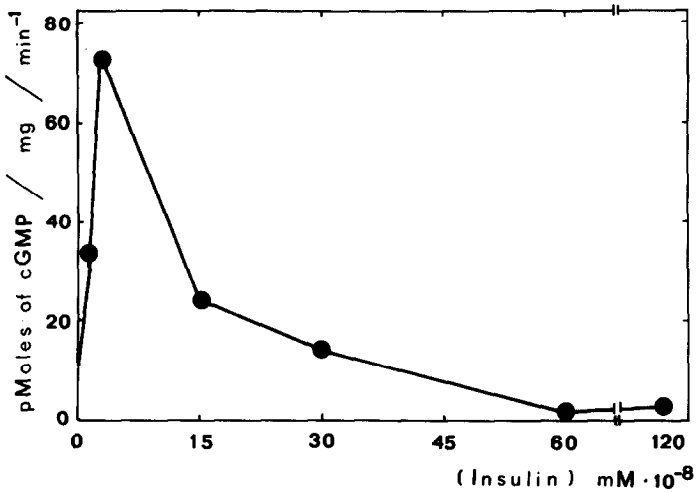

Fig.1: The in VITRO effects of insulin varying concentrations on the rat liver soluble guanylate cyclase activity.
The incubation mixture contained ,in a final volume of 0.1 ml 50 mM Tris-HCl buffer pH 7.6, 4 mM $MnCl_2$, 1 mM GTP, 0.5 mM IBMX and 1.5 μCi of α^{32}P GTP (S.A. 10-50 Ci/mmol),and the indicated insulin concentrations.The reaction was started by the addition of 80-100 μg of membrane proteins carried out for 10 min. at 37°C and stopped by the addition of 100 μl of stopping solution 50 mM Tris-HCl buffer pH 7.6, 3 mM cGMP , 0.1 mM EDTA , ^{3}H cGMP (S.A. 15-20 Ci/mmol) equivalent to 25,000-30,000 cpm for determination of the recovery. The cyclic GMP was isolated through the chromatographic procedure described by Siegel et al (29), slightly modified (See also Methods).

The dose effect relationship, evidenced by the results reported in figure 1, shows that the stimulating effect is strictly linked to the lower concentrations; in fact it is maximally obtained at a concentration of 3×10^{-11}M and decreases at higher concentrations,disappearing at a concentration close to 6×10^{-11}M.

The results of the kinetic experiments depicted in figure 2 , indicate that both insulin and maximally stimulating NaN_3 concentrations increase, to a different extent, the Vmax (change from 0.11 mM /mg protein/min to 0.13 and 0.50 mM/mg protein/min rispectively in presence of Insulin ▲—▲ and NaN_3 ■———■ ,while the apparent Km is decreased in the presence of insulin (it changes from 0.04 to 0.03) but it is increased in the presence of NaN_3 (it changes from 0.04 to 0.08).

Not withstanding the presence of the IBMX inhibitor at the concentrations of 0.5 mM , a significant phosphodiesterase activity on the cyclic GMP was present in the enzymic source we used for the guanylate cyclase activity . These findings prompted us to investigate the effect of insulin on the cyclic GMP phosphodiesterase activity. The results are reported in Table II. They indicate that an insulin concentration (3×10^{-11} M) maximally stimulating the soluble guanylate

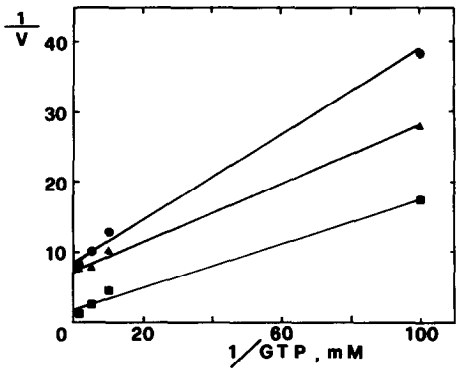

Fig.2: Kinetics of the rat liver soluble guanylate cyclase activity (●——●) control; (▲——▲) in the presence of 3 x 10^{-11} M insulin; (■——■) in the presence of 10 mM NaN_3.
The appearent Km's were corresponding respectively to 0.04 (●——●); 0.03 (▲——▲) and to 0.08 (■——■).
For the other experimental condition , see table I legend and Methods.

cyclase activity , does not affect the PDE activity on the cyclic GMP, either in presence or in absence of 0.5 mM PDE inhibitor IBMX , as well as 10 mM NaN_3 is not effective on the hydrolyzing activity on the cyclic GMP. Only in the presence

Table II

The influence of insulin on the rat liver soluble hydrolyzing activity on cyclic GMP (PDE on cyclic GMP).

Addition	pMoles of cGMP hydrolyzed/mg protein/min with IBMX	without IBMX
None	.87±.07	1.58±.10
Insulin 3.0 x 10^{-11} M	.84±.04	1.61±.07
Insulin 1.5 x 10^{-10} M	.65±.06	1.55±.09
Insulin 3.0 x 10^{-10} M	.69±.08	1.53±.06
NaN_3 10 mM	.87±.09	1.54±.11

The incubation mixture for the hydrolyzing activity on the cyclic GMP contained in a final volume of 0.1 ml: Tris-HCl buffer 10mM (pH 8.0), dithiothreitol 0.2 mM, sucrose 0.25 M, $MnCl_2$ 1.2 mM, creatine kinase 1mg/ml, creatin phosphate 30 mM, cyclic GMP 1x10^{-6} M and ^{3}H cyclic GMP (S.A. 10 Ci/mmol) corresponding to 50,000 cpm was added when added IBMX 0.5 mM; for determination of the activity. The reaction was started by the addition of the enzymic source (100-150 µg of protein) and carried out at 37°C for 10 min, stopped by boiling for 3 min . The formed GMP was hydrolyzed to guanosine throught an enzymic digestion by venom snake 5' nucleotidase according to Thompson et al. (30) and isolated as described in Materials and Methods. For other experimental conditions see Methods.
The results are means ± S.D. of three experiments; each was carried out in triplicate.

of the inhibitor IBMX , the higher insulin concentrations (5×10^{-11} M) appears able to decrease the investigated enzymic activity.

Insulin is able to exert most part of its profound influence on numerous aspects of the cellular metabolism by simply interacting with the surface membrane receptors (22);the existence of some intracellular mediators has been demonstrated although their chemical structure is not yet clear (32,33); they appear to modulate the activity of key enzymic proteins, likely modulating the phosphorylation - dephosphorylation processes that proteins undergo.

Our results indicate that insulin, either directly or through not yet identified cytosolic derivates,can influence the cyclic GMP level by entering the cell. The increase of the intracellular cyclic GMP content appears to be obtained through a stimulation of the soluble guanylate cyclase activity at the insulin lowest tested concentrations, to which also a synergic depression of the cyclic GMP catabolism at a higher insulin concentrations is coupled.

References

1) Butcher R.W., Sneyd J.G.T., Park C.R. & Sutherland E.W.(1966) J.Biol.Chem.241,1651-1653
2) Jungas R.L.(1966) Proc.Nat.Acad.Scie. USA ,56,757-763
3) Butcher R.W., Baird C.E.& Sutherland E.W.(1968) J. Biol. Chem. 243, 1705-1712
4) Jefferson L.S., Exton J.H.,Butcher R.W., Sutherland E.W. & Park R.C. (1968) J.Biol.Chem. 243, 1031-1
5) Manganiello V.C, Murad F., & Vaughan M.(1971) J. Biol.Chem. 254, 2195-2202
6) Exton J.H.,Lewis S.B., Ho R.J.,Robinson G.A., & Park C.R. (1971) Ann.N.Y.Acad.Sci.185, 85-100
7) Illiano G., Tell G.P.E., Siegel M.I., and Cuatrecasas P.(1973) Proc.Nat. Acad. Scie. USA, 70, 2443-2447
8) Baker P.F.,& Carruthers A.(1980) Nature 286, 276-279
9) Hepp K.D.(1971) FEBS Lett. 12, 263-266
10) Illiano G., and Cuatrecasas P.(1972) Science 175, 906-908
11) Hepp H.D.& Renner R.(1972) FEBS Lett. 20, 191-194
12) Hepp K.D.(1972) Eur.J. Bioch. 51, 266-276
13) Flawià M.M., & Torres H.M.(1973) FEBS Lett. 30, 74-78
14) Flawià M.M. & Torres H.M.(1973) J.Biol.Chem. 248, 4517-4520
15) Loten E.G.,& Sneyd J.G.T.(1970) Biochem.J. 120, 187-193
16) Vaughan M.(1972) in Insulin Action ed Fritz I.B.pp 297-318, Academic Press New York USA.
17) Kono T., Robinson F.W., and Sarver J.(1975) J. Biol. Chem 250, 7826-7835
18) Ernest G., Loten F.D., Assimacopoulos-Janet , Exton J.H.and Park C.R.(1978) J.Biol.Chem.253, 746-757
19) Marchmont R.J. & Houslay M.D.(1980) Nature 286, 904-906
20) Makino H., Kono T.(1980) J. Biol. Chem. 255, 7850-7854
21) Shelton Earp H.(1980) J. Biol.Chem.255, 8979-8982

22) Cuatrecasas P.(1969) Proc.Nat. Acad. Scie. USA, 63,450-457
23) Shecter Y., Schlessinger J., Jacobs S., Chang K., and Cuatrecasas P.(1978) Proc Nat. Acad. Scie. USA, 75, 2135-2139
24) Schlessinger J., Schechter Y., Willingham M., &Pastan I. (1978). Proc.Nat. Acad. Scie. 75, 2659-2663
25) Carpentier J.L., Gorden P., Amherdt M., Van Obberghen E., Khan C.R.(1978) J.Clin.Invest. 61, 1056-1070
26) Bergeron J.J.M., Silkstrom R., Hand A.R., & Posner (1979) J. Cell. Biol. 80, 427-443
27) King A.C., and Cuatrecasas P.(1981) New Engl.J.Med. 305, 77-78
28) Fehlman M., Carpentier J.L., Van Obberghen E., Freycht P., Thamm P.,
29) Saunders D., Bradenburg D., and Orci L. (1982) Proc.Nat. Acad. Scie. USA, 79, 5921-5925
30) Siegel M.I., Puca G.A., and Cuatrecasas P.(1976) Bioch.Bioph. Acta 438, 310-323
31) Thompson W.J. & Appleman (1971) Biochemistry 10, 311-316
32) Desbuquois B., Krug F., and Cuatrecasas P.(1974) Bioch. Bioph. Acta 343, 101-102
33) Saltiel A.R., Siegel M.I., Jacobs S., and Cuatrecasas P.(1982) Proc. Nat. Acad. Scie. USA 79, 3513-3517
34) Saltiel A.R., Doble A., Jacobs S., and Cuatrecasas P.(1983) Biochem. Bioph. Res. Comm. 110, 789- 795