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BIOMEMBRANE COOPERATIVE ENZYMES

IN VIVO MODULATION OF RAT ERYTHROCYTE ACETYLCHOLINESTERASE BY INSULIN IN NORMAL AND DIABETIC CONDITIONS

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Summary

The present study investigated the effect of insulin in vivo on the changes in the cooperativity of a membrane-bound enzyme. The allosteric inhibition by F^- of the erythrocyte membrane acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) was studied during intravenous glucose tolerance tests in control and alloxan-induced diabetic rats. In the former group, the value of n decreased from 1.6 to 1.0 whereas it remained about 1.6 in the latter groups. Intravenous injection of insulin (30 U/kg) decreased the values of n in both groups. It is suggested that the in vivo insulin action on membrane cooperative enzymes could also take place in insulin target cells.

Introduction

Previous reports from this laboratory indicated that the cooperative behavior of several membrane-bound enzymes could be used as a tool to detect modifications at the cell membrane level [1]. This novel approach was illustrated in the case of cholesterol [2], organophosphorus compound [3] and also with several hormones [4—8], e.g. it was shown that insulin at physiological plasma levels affects these membrane-bound systems [4,5]. This action was detected in vitro through cooperative changes in the rat erythrocyte membrane-bound acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) and $(Na^+ + K^+)$-ATPase [4], and strikingly in *Escherichia coli* membrane-bound Ca^{2+}-ATPase

[5]. Since the membrane from rat erythrocytes and *E. coli* differ greatly in properties, functions and composition a general action of insulin on membrane cooperative enzymes through changes in the membrane conformation was suggested [5]. The present communication provides the first evidence for an in vivo modulation of the allosteric properties of rat erythrocyte acetylcholinesterase by insulin.

Material and Methods

Male Sprague-Dawley rats (220—330 g) were used. After weaning, the animals were grown on a basic diet supplemented with corn oil to obtain erythrocyte membranes with high fatty acid fluidity and high values of n for the allosteric inhibition by F^- of the membranous acetylcholinesterase [9]. The correlation between the membrane fluidity expressed as the ratio double-bond index/saturated fatty acid and the values of n was highly positive ($r = 0.90$) [9]. This correlation was demonstrated in studies carried out with rats fed with different fat-supplemented diets.

Intravenous glucose tolerance tests were performed on normal and diabetic animals fasted 20—24 h and anesthetized with sodium pentobarbital (50 mg/kg body weight). Glucose (0.5 g/kg of body weight) was administered in a 50% solution intravenously (caudal vein) to circumvent the influence of the gastrointestinal hormones [10,11]. Thereafter, two blood samples were taken by cardiac puncture for plasma glucose and kinetic constant determinations. The first sample was taken at zero time while the second one was drawn later on, as indicated in Fig. 2. To avoid the stress resulting from blood removal, separate animals were employed.

Diabetes was induced by injection of alloxan (200 mg/kg) [12] and confirmed by determination of hyperglycemia after 3 or 4 days of the administration of the drug. The animals were used at this time for the experiments summarized in Table II. The plasma glucose concentration for the 12—16 h fasted alloxan-treated rats ($n = 6$) was 605 ± 101 mg/100 ml. These data are in agreement with those reported by other workers [12]. Porcine insulin was administered intraperitoneally (30 U/kg) and blood samples withdrawn 45 min after injection.

Details concerning erythrocyte ghost preparation, assay of acetylcholinesterase activity and calculation of kinetic parameters were given in preceding papers [4,9]. Acetylcholinesterase activity was determined without including $MgCl_2$ in the reaction medium. Neither the specific activity nor kinetic parameters were modified by the absence of $MgCl_2$. In some experiments, inhibition by F^- of the acetylcholinesterase was studied with red cells [3]. Plasma glucose concentration was measured colorimetrically [13]. Guinea-pig anti-porcine insulin serum (Wellcome Co., U.K.) was used to neutralize rat plasma insulin. It is assumed that this antibody behaves in the same way against porcine or rat insulin [14].

Results

As can be seen in Fig. 1, the value of the Hill coefficient, n, for the inhibition by F^- of the erythrocyte membrane-bound acetylcholinesterase from rats

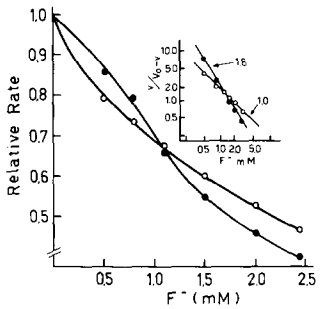

Fig. 1. Inhibition by F^- of the membrane-bound acetylcholinesterase in the absence (●———●) and in the presence (○———○) of $1 \cdot 10^{-9}$ M insulin. Inset shows Hill plots of the same data. The same enzymatic preparation was used for the control and hormone test. When two different curves are made with the same enzymatic preparation under the same enzymatic conditions, the individual points and the slopes (n values) obtained show a maximum variability of 5%

fed a corn oil diet was changed from 1.6 to 1.0 by the presence of insulin in the enzymatic assay medium. Taking into account the positive relationship between the n values for the acetylcholinesterase and membrane fluidity, this change suggests that insulin decreases membrane fluidity obviously without modifying its fatty acid composition. Additional experimental support for this concept has been given in previous papers [1,4,5]. The effect of insulin persisted in the isolated membrane from whole red cells preincubated with insulin 138 μU/ml (10^{-9} M) even though several washes were performed to obtain membrane preparations. Intraperitoneal injection of insulin also decreased the n values. Table I summarizes these results.

It is known that the basal glucose levels in alloxan-induced diabetic rats are higher than in control animals and that, during a glucose tolerance test, the glucose levels increase in both groups of animals. However, insulin levels increase significantly only in normal rats [10]. Plasma glucose concentrations from these rats are shown in Fig. 2 and in Table II and are within the ranges reported by other workers [11,15]. As can be seen in Fig. 2, the values of n decrease in control rats 10—20 min after glucose injection. The Hill coefficients returned to their original values between 2 and 6 h after glucose administration. The values of n did not change when a glucose-free solution was injected into control rats (not shown).

TABLE I

EFFECT OF INSULIN ON THE VALUES OF n IN ACETYLCHOLINESTERASE OF DIFFERENT ERYTHROCYTE MEMBRANE PREPARATIONS FROM CONTROL RATS

n values are mean of 3—5 rats/group ± S.E. Values followed by different letters were significantly different ($P < 0.001$) when compared by the Student's t-test.

Enzymatic preparation	n values
Membrane	1.53 ± 0.05 [a]
Membrane + insulin in vitro (138 μU/ml)	1.03 ± 0.02 [b]
Membrane from whole red cell preincubated 15 min with insulin (138 μU/ml)	1.05 ± 0.03 [b]
Membrane from rats injected with insulin	0.95 ± 0.10 [b]

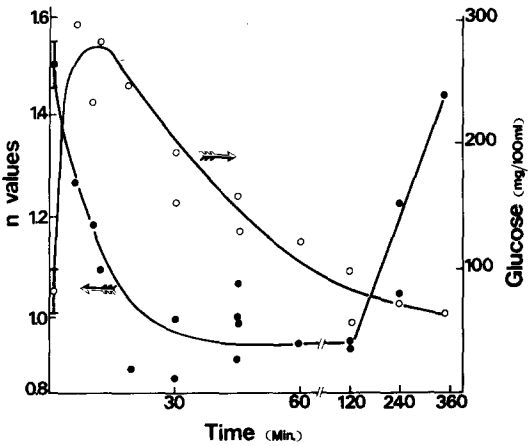

Fig. 2. Hill coefficient (n) and glucose concentration as a function of time after glucose injection to control rats. At zero time the values of $n \pm$ S.E. and glucose concentration ±S.E. are given for all the experimental animals used (16 rats). At different times after glucose injection the individual values of Hill coefficient (●) and glucose concentration (○) are shown.

TABLE II

CHANGES IN THE VALUES OF n AND GLUCOSE CONCENTRATION IN ALLOXAN-INDUCED DIABETIC RATS

Expt.	Red cell from diabetic rats treated with:	Plasma glucose (mg/100 ml)	n values
I	None	449 ± 35	1.53 ± 0.11
	Glucose injection *	994 ± 30	1.55 ± 0.10
II	None	500 ± 150	1.53 ± 0.09
	Insulin injection *	450 ± 100	1.00 ± 0.07
III	None	620 ± 97	1.50 ± 0.03
	None + insulin in vitro (138 μU/ml)	—	0.85 ± 0.12

* Blood samples were taken after 20 or 45 min after glucose or insulin injection, respectively.

TABLE III

EFFECT OF PLASMA INSULIN LEVELS AND ANTI-INSULIN SERUM ON CHANGES OF n VALUES

After preincubation for 15 min, the cell were washed twice with sodium phosphate buffer (155 mM, pH 8.0) and the values of n were determined. n values are mean of 3—5 rats/group ± S.E. Values followed by different letters were significantly different ($p < 0.001$) when compared by the Student's t-test for paired samples.

Red cells from control rats preincubated with	n values
Plasma from glucose-loaded control rat	1.00 ± 0.02 [b]
Plasma from glucose-loaded control rat * + anti-insulin serum **	1.55 ± 0.05 [a]
Plasma from control rat + anti-insulin serum **	1.53 ± 0.05 [a]
Plasma from control rat	1.55 ± 0.04 [a]
Plasma from control rat + insulin (100 μU/ml)	0.93 ± 0.10 [b]

* Blood sample was taken 20 min after glucose injection.

** The reaction between plasma and anti-insulin serum was carried out by 24 h at room temperature. Plasma without addition of anti-insulin was incubated under similar conditions as control. This last incubation did not alter the respective plasma action on n values.

The Hill coefficients were not modified during glucose tolerance tests in diabetic rats. When these rats were treated with insulin, the n values decreased from 1.5 to 1.0. However, the plasma glucose levels did not decrease after 45 min of intraperitoneal hormone administration, in agreement with previous observations [16]. The presence of insulin in the enzymatic medium also caused changes in the values of n. Table II summarizes these observations.

Results in Table III confirm the relationship between the increase in plasma insulin levels and changes in the n values. The preincubation of red cells from control rats with plasma from glucose-loaded animals before n determinations changed the values of n from 1.5 to 1.0. The specific anti-insulin serum blocked this action. In addition, changes in the values of n were observed when insulin (100 μU/ml) was added to the plasma obtained from control rats to raise its concentration to the levels found in plasma from glucose-loaded rats [11].

Discussion

Our previous paper [4] showed that insulin in vitro modifies the cooperativity of the membrane-bound acetylcholinesterase. This insulin action is specifically blocked by glucagon [7]. The action of insulin on a membrane cooperative enzyme was detected here in vivo in control rats during intravenous glucose tolerance test or during insulin injection. Changes in the values of n of the acetylcholinesterase were observed in conditions in which plasma insulin levels increased [16]. Anti-insulin serum was completely effective in reversing the effect of plasma from glucose-loaded control rats on n values. (Table III).

In diabetic rats, during the intravenous glucose tolerance test the changes in the membrane-bound system mediated by insulin did not take place. This is likely to be due to the fact that the hormone plasma concentration remains at constant low levels [11]. The pathological implications of this observations under diabetic conditions deserve further study. Insulin in vivo (Expt. II, Table II) and in vitro (Expt. III, Table II) in diabetic rats caused changes in the n values, indicating that no alteration was produced in membrane acetylcholinesterase system in diabetic rats.

This work suggests that the erythrocyte membrane respond to changes in the plasma insulin levels in vivo. The plasma insulin concentration changes from 15—60 to 180—300 μU/ml within 4—12 min after glucose injection [11,15]. In experiments carried out in vitro, insulin at 120 μU/ml ($8.5 \cdot 10^{-10}$ M) or more gave maximal effects on the values of n of the red cell membrane-bound acetylcholinesterase [4]. It must be emphasized that there is suggestive agreement between the range of insulin concentration in vitro [4,5] and in vivo and that in which the effect on the Hill coefficient is observed. It is interesting to note that in bacterial systems the half-maximal effect for insulin was obtained at 140 μU/ml [5].

It was suggested earlier that the metabolic effects caused by insulin are based on the propagation of effects resulting exclusively from specific interactions with the membrane of the target cell [17]. Plasma membrane vesicles isolated from insulin-treated adipocytes of the rat epididymal fat pad retained an accelerated uptake and release of glucose when compared to similar preparations from cells not exposed to the hormone [18]. Direct addition of insulin to

the membrane produced no effect; exposure of cells prior to rupture was required. The effect of insulin on glucose transport does not involve large scale changes in the structure of the membrane evaluated by physical methods [18]. The papers dealing with physicochemical methods for the study of intact membrane stress the fact that these methods are not sensitive enough to detect specific changes because their results are statistical, that is, the measurements reflect the overall contributions of all proteins and/or of the bilayer as a whole [19–21]. In our case, the effect of insulin persisted in both membrane preparations isolated from whole red cells exposed to hormone and red cells obtained from rats in which the physiological level of insulin in plasma was raised. On the other hand, in contrast to adipocyte systems, we were able to observe changes in the values of n of several unrelated membrane-bound enzymes [4,5] by addition of insulin to enzymatic medium containing non-treated membranes. This difference could be explained by taking into account that we used a membrane-cooperative enzyme system as a probe to detect influences of the environment on a membrane in which the enzyme is embedded. Variations in the interaction energies between membrane enzyme as low as 700 cal/mol would be enough to trigger a significant change in the Hill coefficient (allosteric 'probes') [22].

Erythrocytes contain specific insulin binding sites [23]. Under special conditions, erythrocytes are responsive to insulin [24–26]. Nevertheless, erythrocytes are usually classified as non-responsive to insulin. Under physiological conditions, the significant metabolic role of insulin lies in tissues other than in red cells [27]. In a non-responsiveness cell the insulin receptor may be present and the molecular interaction with insulin may occur, but the transduction system or the enzymatic machinery required for manifestation of an overt metabolic response may be absent [27]. The determination of the changes in the Hill coefficient from membrane-bound enzymes seems to bypass many of these complications in insulin action, because the action is directly on membrane in which the natural probes are immersed. Insulin did not modify cooperative properties of the solubilized enzymes [4,5]. However, the relationship between the participation of the insulin receptor and changes of n values of the membrane-bound enzymes observed remains to be determined. An effect of cold exposure and of thyrotropin-releasing hormone injection on the allosteric properties of membrane-bound acetylcholinesterase has been described [28]. This and the present work illustrate the use of a novel enzymatic method for detecting changes in vivo in a membranous system.

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