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Activation and desensitization of glycolysis by stimulation of adenylate cyclase in rat reticulocytes

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Isoproterenol or forskolin induce a 10–15-fold increase in concentration of cyclic AMP in rat reticulocytes as compared with the basal level of 2.3 ± 0.3 μM. Glycolysis is stimulated by both compounds transiently more than 2-fold with a peak after 7.5 min followed by an exponential decline. The glycolytic rate in the presence of 10 μM isoproterenol or 10 μM forskolin did not return to basal levels within 60 min of incubation, but was depressed by as much as 50% under the influence of 100 μM forskolin. This phenomenon is designated as metabolic desensitization. The stimulation of glycolysis is probably due to activation of phosphofructokinase as well as to stimulation of Na^+,K^+-ATPase. The diminished glycolytic flux during the period of metabolic desensitization is accompanied by a decline of glucose 6-phosphate and in the presence of high concentrations of forskolin also by a decrease in glucose 1,6-bisphosphate. A lower rate of influx of glucose is postulated.

cyclic AMP; Glycolysis; Isoproterenol; Forskolin; Na^+,K^+-ATPase; (Rat reticulocyte)

1. INTRODUCTION

In a previous study it was shown that the β-adrenergic agonist isoproterenol produces an accumulation of cyclic AMP and stimulates transiently glycolysis of rat and rabbit reticulocytes, without any change in oxygen consumption [1]. The present study was designed to answer the following questions:

(i) Can forskolin, a potent stimulator of adenylate cyclase of a variety of cells [2–4], mimic the effect of isoproterenol on glycolysis of reticulocytes?

(ii) What is the dose-response relationship with respect to stimulation of cyclic AMP and glycolysis for both isoproterenol and forskolin?

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(iii) What are the causes of the stimulation of glycolysis; does activation of Na^+,K^+-ATPase play a role?

(iv) What is the precise time course of the stimulated glycolysis?

2. MATERIALS AND METHODS

(−)-Isoproterenol was obtained from Sigma and forskolin from Calbiochem. Glucose 1,6-bisphosphate was from Boehringer. Rat reticulocytes were prepared as previously described [1]. Male Wistar rats were injected during 3 consecutive days with neutralized 2% phenylhydrazine hydrochloride solution (35 mg/kg). On the 7th day after the first injection the blood contained 86 ± 4% reticulocytes at hematocrit values of 0.2–0.3. The reticulocytes were incubated in their own plasma adjusted to a final packed cell volume of 0.20 with isotonic buffer containing 50 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4

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and 10 mM glucose at 37°C. After 5 min of preincubation 0.1 mM isobutylmethylxanthine was added and the cells were further incubated with or without isoproterenol or forskolin.

Samples for determination of glucose, lactate, glycolytic intermediates and cyclic AMP were taken at the time intervals indicated in the figures. The extraction was carried out by addition of 1 vol. of 0.6 N perchloric acid and neutralization was performed as in [5]. Glucose, lactate and glycolytic intermediates were assayed enzymatically according to the methods described in [6]. Cyclic AMP was determined by the protein-binding assay [7]. Glucose 1,6-bisphosphate content was measured as cofactor of phosphoglucomutase as in [8] by a spectrophotometrical method [9].

3. RESULTS AND DISCUSSION

In fig.1 are presented the dose-response relationships of rat reticulocytes with respect to the accumulation of cyclic AMP after 7.5 min of incubation at 37°C for both isoproterenol and forskolin in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine. The unstimulated reticulocytes, directly resuspended in the incubation medium and immediately sampled, yielded a value of 2.28 ± 0.34 nmol (n = 11) cyclic AMP per ml packed cells. It may be seen (fig.1A) that the curve for isoproterenol follows a sigmoid course with a maximum of about 20 nmol cyclic AMP/ml cells produced by 10 μM of the agonist. Half-maximal activation was observed at 0.5 μM isoproterenol. Forskolin (fig.1B) induced an even greater increase in cyclic AMP, without any indication of a plateau. It should be mentioned that with the highest concentration of forskolin used (100 μM) an uncoupling of respiration was observed.

The time courses of accumulation of cyclic AMP for 10 μM isoproterenol and for 10 and 100 μM forskolin are shown in fig.2. It may be seen that with isoproterenol a plateau of cyclic AMP was reached after 7.5 min, which was maintained over the entire period of observation of 60 min. An equimolar concentration of forskolin exerted a slower effect, but produced the same plateau of cyclic AMP after 15 min. With the high concentration of forskolin a plateau nearly twice as high as

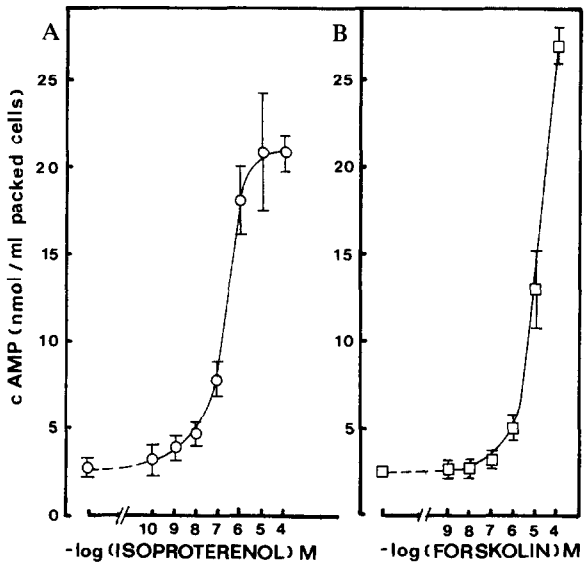

Fig.1. Dose-response curves for isoproterenol and forskolin for the stimulation of cyclic AMP formation in rat reticulocytes. Cells were incubated in the presence of 0.1 mM isobutylmethylxanthine (IBMX) at the indicated concentrations for 7.5 min. The basal concentration of cyclic AMP was determined to be 2.3 ± 0.4 nmol/ml packed cells. The values are means ± SE of five separate experiments measured in triplicate. All differences in the concentration of cyclic AMP were statistically significant from controls with IBMX alone for concentrations exceeding 10^{-8} M isoproterenol or 10^{-6} M forskolin.

with isoproterenol was produced after about 30 min.

One may conclude that forskolin is a more potent stimulator of adenylate cyclase than isoproterenol. In fig.3 are presented the effects of isoproterenol and forskolin on glucose consumption (panel A) and lactate production (panel B). It may be seen that isoproterenol induced an increase of up to 239 ± 14% of glucose consumption and of 235 ± 20% of lactate formation, after 7.5 min of exposure, as compared with control values. This stimulation was antagonized by propranolol as reported previously [1]. Forskolin at both low (10 μM) and high concentration (100 μM) produced a similar excursion of glycolysis, with values of 226 ± 22 and 237 ± 25% of glucose and 234 ± 14 and 218 ± 27% of lactate consumption, respectively. The peaks of glycolytic activation occurred

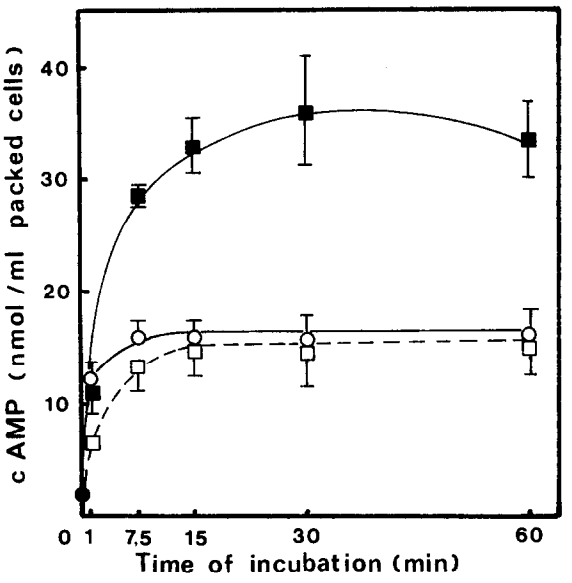

Fig.2. Time course of cyclic AMP accumulation in rat reticulocytes. The basal cyclic AMP (●) did not change in the controls during incubations with IBMX up to 60 min. Reticulocytes were incubated with 10 μM isoproterenol (○—○), 10 μM forskolin (□---□) and 100 μM forskolin (■—■). Each point represents the mean ± SE of five experiments.

at the same time point for all three conditions. It can also be seen that a decline of glycolytic rates took place despite the maintenance of high concentrations of cyclic AMP (see fig.2). Whereas the glycolytic rates with isoproterenol and the low concentration of forskolin had not yet returned to the control value after 60 min, with the high concentration of forskolin the glycolytic rate fell drastically to values as low as 50% of the initial rate. The greater decline of glucose consumption as compared with lactate production is noteworthy. This decline of glycolysis despite the persistence of high cyclic AMP concentrations may be considered as a desensitization of the glycolytic system which has become refractory to the stimulatory effect of cAMP.

What is the cause of the rapid stimulation of glycolysis by increased cellular cyclic AMP? For this purpose the changes of glycolytic intermediates were determined after varying time periods of incubation with isoproterenol. A cross-over plot in which the values after 7.5 min are compared with control values is presented in fig.4. A strong decline of the hexose monophosphates indicates a relative activation of the flux through phosphofructokinase and also increased hexokinase activity owing to the decreased product inhibition by glucose 6-phosphate. The cross-over at the pyruvate kinase is not easily explained. The expected increase in ADP concentration owing to stimulation of ATP-consuming processes was not observed. Instead, all adenine nucleotides decreased (see [1] and data not shown). The cross-over plots after longer periods of time, up to 60 min after exposure to isoproterenol, presented similar patterns (not shown).

A logical and easily tested candidate for an ATP-consuming process which may have been stimulated is the Na^+,K^+-ATPase. In fig.5 are presented the effects of ouabain, a well-known inhibitor of Na^+,K^+-ATPase, on lactate formation induced by isoproterenol and forskolin. It may be seen that the stimulation of glycolysis was depressed to one-half or less if the Na^+,K^+-ATPase was inhibited; thus stimulation of Na^+,K^+-ATPase is a significant factor in the metabolic response of the reticulocyte to stimulation of its adenylate cyclase. Two possibilities for the stimulation of Na^+,K^+-ATPase may be envisaged: firstly, a direct activation of the enzyme, perhaps by cyclic AMP-dependent phosphorylation and secondly, an increased influx of Na^+ with resulting higher activity of ion transport.

What might be the reason for the metabolic desensitization observed at persistently high cyclic AMP levels? The reduction of glycolysis implies a decreased activity of hexokinase. Since glucose 6-phosphate, one of the inhibiting metabolites, declined, we looked for changes in glucose 1,6-bisphosphate, which is known to be one of the main inhibitors of hexokinases [10]. The data on changes of this metabolite under the influence of forskolin are presented in fig.6. It may be seen that 10 μM of this compound produced a minor drop and 100 μM a large decrease in concentration of glucose 1,6-bisphosphate from the initial level of 133 ± 9.9 nmol/ml cells during the incubation of rat reticulocytes. For comparison the decline of glucose 6-phosphate is also shown in fig.6. Thus, one may conclude that both main cellular inhibitors of hexokinase decrease under conditions of stimulation of adenylate cyclase. The logical reason for a decreased glycolytic flux appears to be

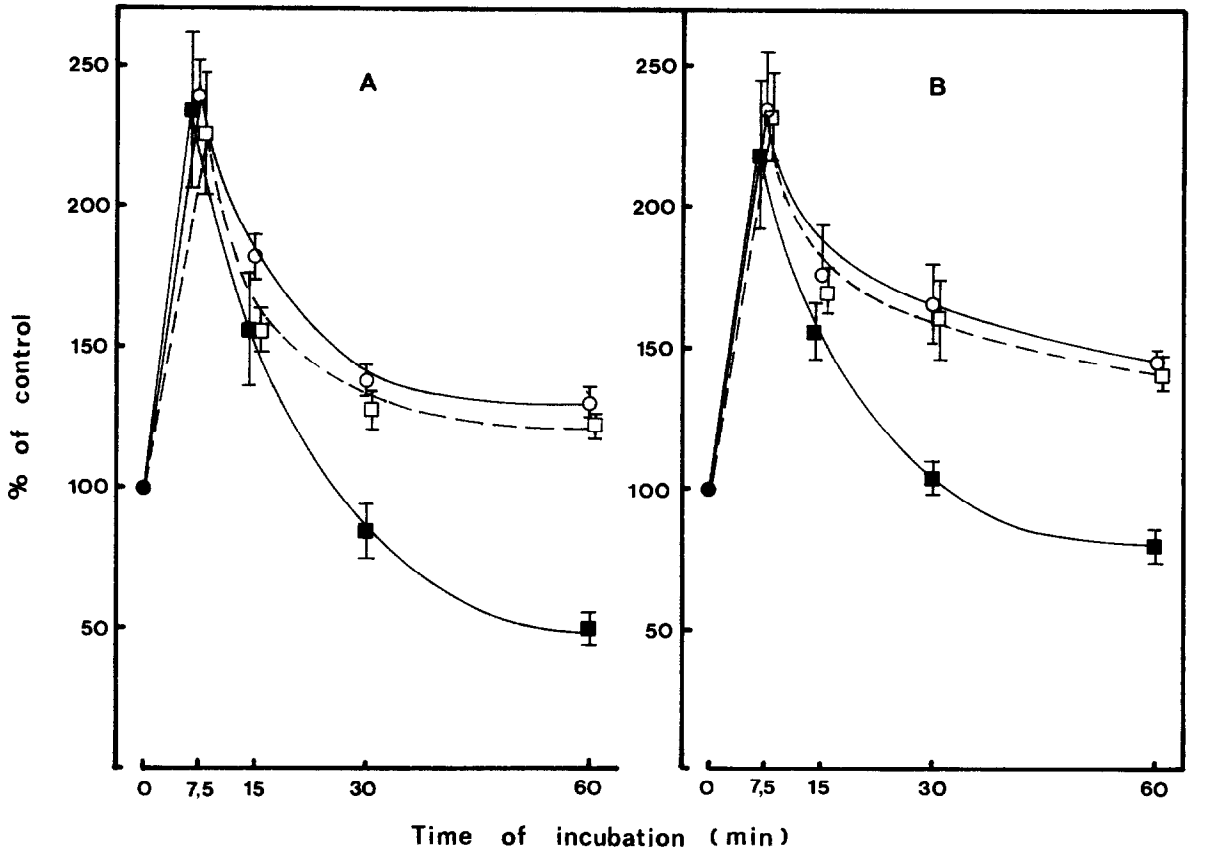

Fig.3. The effects of isoproterenol and forskolin on glucose consumption (A) and lactate production (B) of rat reticulocytes. The glycolytic rates in control incubations taken as 100% were: 7.3 ± 0.54 μmol/ml cells per h glucose consumption and 11.96 ± 0.71 μmol/ml cells per h lactate production. Each point represents the mean ± SE of seven incubations for 10 μM isoproterenol, of five for 10 μM forskolin and of three for 100 μM forskolin. Symbols and concentrations are the same as in fig.2.

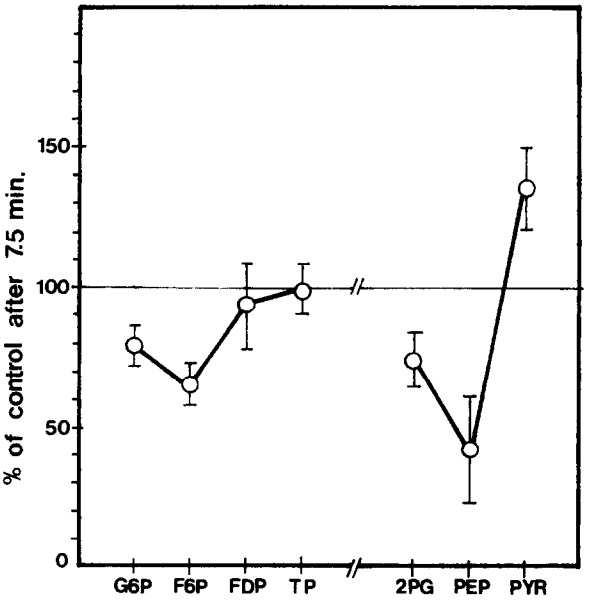

Fig.4. Changes of glycolytic intermediates after 7.5 min of incubation of rat reticulocytes with isoproterenol (10 μM) compared to controls (100%). The values with error bars represent means ± SE of seven experiments. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-bisphosphate; TP, triose phosphates; 2PG, 2-phosphoglycerate; PEP, phospho*enol*pyruvate; PYR, pyruvate.

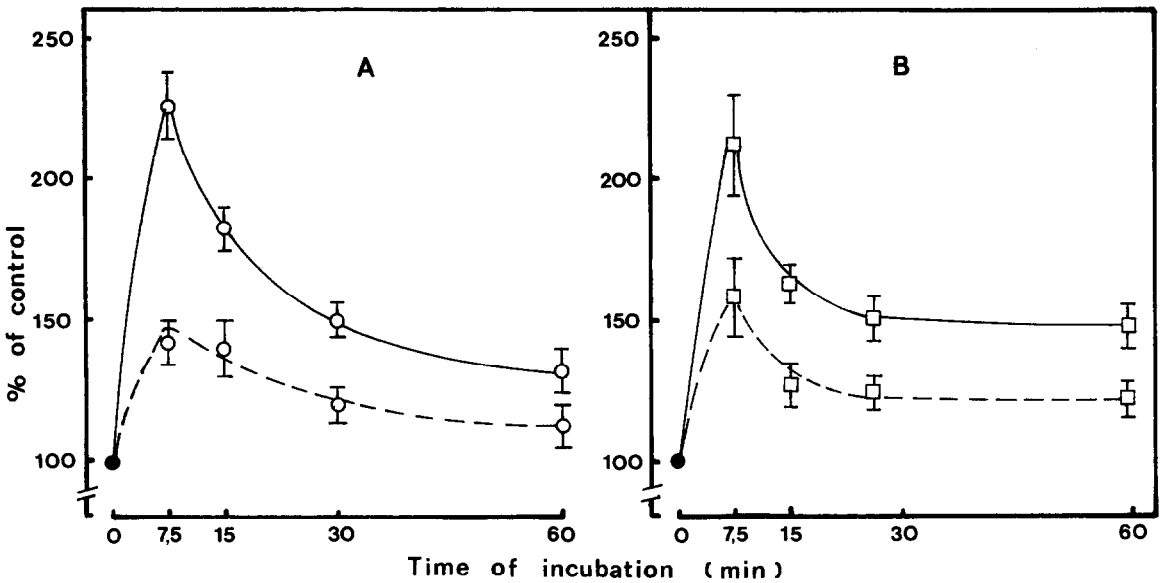

Fig.5. Effect of Na^+,K^+-ATPase inhibition by ouabain on the increased lactate production induced by isoproterenol (A) and forskolin (B). Reticulocytes were incubated with 10 μM isoproterenol without (○—○) and with 0.2 mM ouabain (○---○) in (A) and with 10 μM forskolin without (□—□) and with 0.2 mM ouabain (□---□) in (B). The inhibitory effect of ouabain alone on the lactate production of rat reticulocyte was measured in separate control experiments and amounted to less than 15% decrease of the glycolytic rate. The points in (A) represent means ± SE of seven incubations and of five in (B).

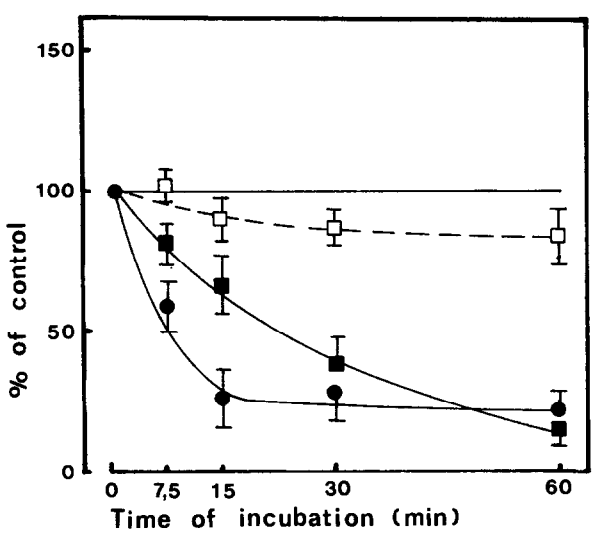

Fig.6. Decline of the content of glucose 6-phosphate (●) and glucose 1,6-bisphosphate (■) in rat reticulocytes in the presence of 100 μM forskolin. For comparison the percent changes are shown for glucose 1,6-bisphosphate at 10 μM forskolin. The concentrations at zero time were taken as 100% for glucose 6-phosphate 171 ± 15 nmol/ml cells and for glucose 1,6-bisphosphate 133 ± 9.9 nmol/ml cells measured in triplicate.

a lower influx of glucose into the reticulocytes. The relatively greater decline of glucose consumption as compared with lactate production in the presence of 100 μM forskolin (see fig.3) is in line with this assumption. As a matter of fact, it has been demonstrated that forskolin exerts a potent inhibitory effect on the transport of monosaccharides in human red cells [11].

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