INITIAL LAG-PHASE IN THE ACTION OF INSULIN ON GLUCOSE TRANSPORT AND cAMP LEVELS IN FAT CELLS

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1. Introduction

Whereas insulin binding to plasma membrane receptors is a well-studied phenomenon, comparatively little is known about the transmission of the hormone signal from the receptor to enzyme systems associated with the plasma membrane. The isolated fat cell lends itself well as a model to the assessment of insulin action upon two major membrane functions, glucose transport and control of cAMP levels. It is not clear whether the transmission of the hormone signal occurs through a common initial mechanism or by direct coupling of hormone–receptor complexes to enzyme systems. Comparison of dose–response curves of glucose oxidation and antilipolysis [1] suggests different mechanisms of action. Here, the initial kinetics of insulin binding are compared with membrane functions between 5 s and 4 min after the first contact of the hormone.

2. Materials and methods

Fed male Wistar rats (180–200 g) were used throughout. Fat cells were prepared as in [2]. All incubations were performed in Krebs-Ringer–Hepes buffer [3] containing 2.5 g/dl crystalline bovine albumin (Behring, Marburg).

2.1. *Binding of [^{125}I]insulin*

Fat cells (4.5–5.5 × 10^5/ml) were incubated in polyethylene vials under constant magnetic stirring at 37°C. [^{125}I]Insulin was added at 25–500 μU/ml. Aliquots of 400 μl were transferred to polyethylene centrifuge tubes which were fitted with an adapter into the rotor of a high-speed table centrifuge (Eppendorf, Model 3004). Separation of cells was performed by centrifugation at 10 000 × *g* × 1 min through dinonylphthalate [4]. The binding reaction was considered to be terminated at the start of centrifugation.

2.2. *Kinetics of cAMP*

Cells (2 × 10^5/ml) were incubated at 37°C under constant stirring in the presence of 1 μM isoproterenol, insulin was added simultaneously or 3 min later. At time points indicated 2 ml samples were removed and the reaction was stopped by mixing with ice-cold trichloroacetic acid (final conc. 5%). Each sample was sonicated (Braun Sonic 125, 20 s, step 60) and centrifuged at 40 000 × *g* × 10 min at 4°C. Trichloroacetic acid was removed by ether extraction (3 × 7 vol.), the samples were concentrated by lyophilisation, and cAMP was determined as in [5] using a test combination provided by the Radiochemical Centre, Amersham.

2.3. *Determination of 3-O-methylglucose transport*

Cells (5 × 10^6/ml) were equilibrated at 37°C. Insulin (25–10 000 μU/ml) or buffer was added, the suspension was agitated and incubated for 10–120 s. At the time points indicated 100 μl samples were drawn together with 200 μl 3-*O*-methylglucose (final conc. 500 μmol/l, 0.1 μCi 3-*O*-[1-^{14}C]methylglucose as tracer) into a mixing pipet (Gilson Medical Electronics, France). After 15 s cells and medium were separated by centrifugation through dinonylphthalate. The cell layer was dissolved in 200 μl diphenylethylamine and counted in 10 ml scintillation fluid

(Szintigel, Roth, Karlsruhe). The extracellular concentration of 3-*O*-methylglucose in the pellet was extrapolated from the kinetic of the initial uptake of 3-*O*-methylglucose. Initial kinetics were established for each experiment. The values for extracellular 3-*O*-methylglucose thus obtained correspond to calculations of the extracellular waterspace from [^{14}C]inulin determinations.

3. Results

3.1. *Insulin binding*

Figure 1 shows the association kinetics of labelled insulin of one specific activity at four different concentrations. The rate of association and the amount bound at equilibrium (30 min) are proportional to the hormone concentration. No initial lag-phase is apparent.

3.2. *Kinetics of cAMP*

Addition of 1 μM isoproterenol leads to a rapid increase in the levels of cAMP with the formation of a plateau after 90 s (fig.2). Concomitant addition of insulin (100 μU/ml) does not change the initial rise for 30–40 s. After this lag-phase the increase is rapidly stopped and a new steady state is reached at ~3 min, the level of which is proportional to the concentration of insulin. This lag-phase remains the same with a higher concentration (500 μU/ml). The same delay is observed when insulin (100 μU/ml) is added 3 min after the start of the incubation with isoproterenol (fig.3). If a submaximal 25 μU/ml is used, the lag-phase is prolonged to 60 s. It was earlier observed that in contrast to insulin, propranolol leads to an immediate effect under similar experimental conditions [6]. Thus the prompt response to isoproterenol and propranolol rules out a delay in mixing as a possible explanation for the lag-phase.

3.3. *Transport of 3-O-methylglucose*

Previous incubation of fat cells with insulin results in an increased glucose uptake [7]. Our results with 3-*O*-methylglucose confirm the observation that insulin increases V_{max} (1.83 nmol/10^5 cells × min) by a factor of 4 without changing the K_m for glucose (about 6 mM) [3,8–10]. Figure 4 demonstrates a similar delay as observed in the case of insulin action on cAMP. Again the lag-phase is about 30–40 s while a maximal effect of insulin is reached at about 100 s.

In both systems, cyclic AMP turnover and glucose transport, the lag-phase is of the same duration, it is

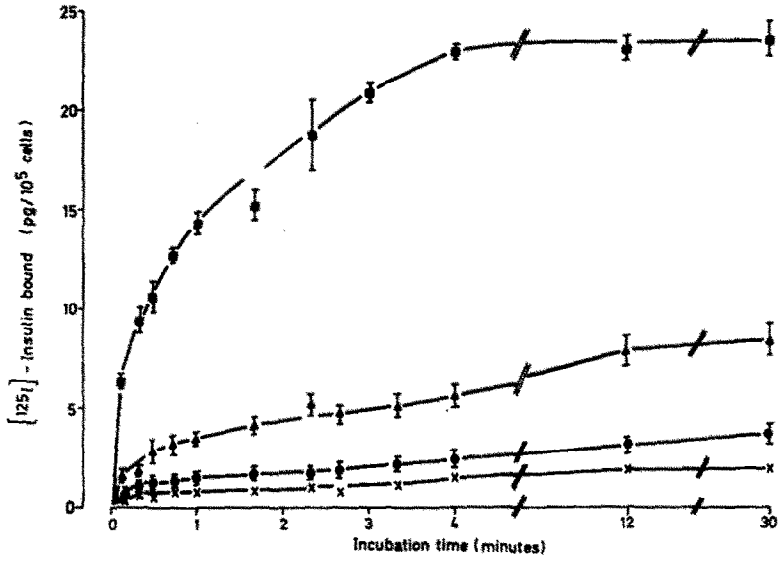

Fig.1. Kinetics of [^{125}I]insulin binding to isolated fat cells. [^{125}I]Insulin in the test was: 25 μU/ml (×–×); 50 μU/ml (●——●); 100 μU/ml (▲——▲); 500 μU/ml (■——■). Values indicated are the mean of 2–4 experiments ± SEM.

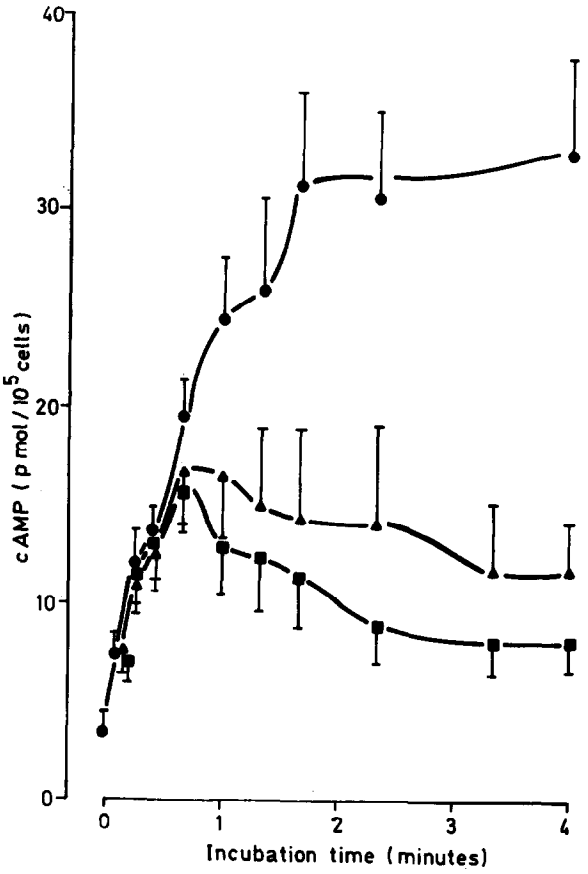

Fig.2. Kinetics of cAMP in fat cells. Effect of isoproterenol (10^{-6} M) (●——●); isoproterenol (10^{-6} M) + insulin (100 μU/ml) (▲——▲); isoproterenol (10^{-6} M) + insulin (500 μU/ml) (■——■). All hormones were added at t_0. Values indicated are the mean of 3–5 experiments ± SEM.

dose-dependent with a maximum at insulin concentrations which gave a maximal biological response (fig.5). Its temperature dependency is illustrated by a 2.4-fold increase at 25°C.

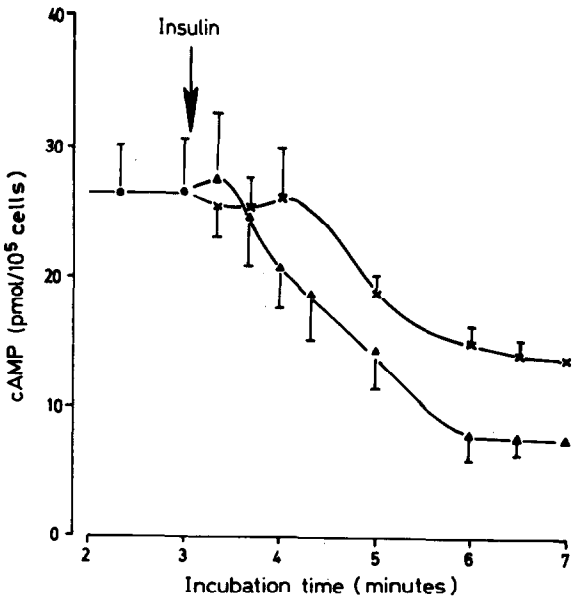

Fig.3. Kinetics of cAMP in fat cells. Effect of isoproterenol (10^{-6} M) + insulin (100 μU/ml) (▲——▲); and isoproterenol (10^{-6} M) + insulin (25 μU/ml) (X–X). Isoproterenol was added at t_0, insulin 3 min later. Values are the mean of 3–6 experiments ± SEM.

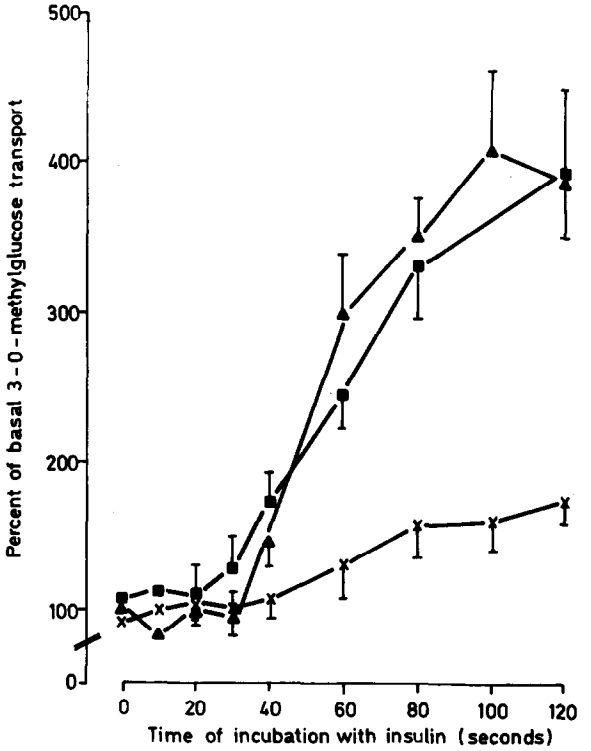

Fig.4. Time course of the insulin effect on glucose transport. Rate of stimulation is plotted versus the time cells are incubated with insulin: 25 μU/ml (X–X); 100 μU/ml (▲——▲); and 500 μU/ml (■——■). Values are the mean of 5–6 experiments ± SEM.

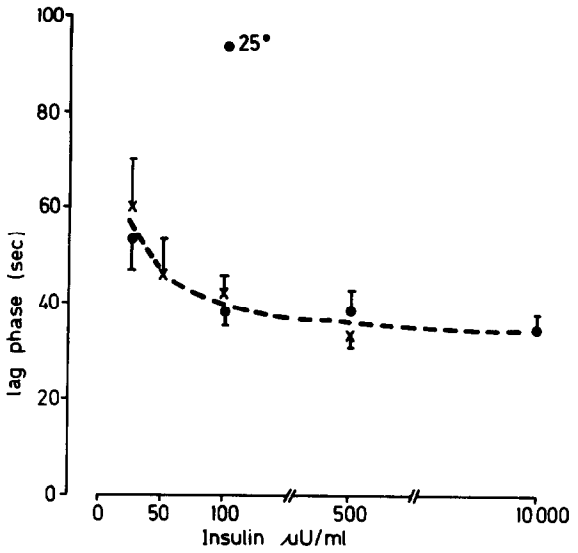

Fig.5. Time lag of the insulin effect on glucose transport (• – •) and cAMP (X – X) as a function of the insulin concentration used in the test. Means of 3–10 experiments ± SEM.

4. Discussion

The binding of insulin to its receptor sites is a fairly rapid phenomenon, and an initial time lag cannot be distinguished. The rate of association is proportional to the concentration of [^{125}I]insulin used. On the other hand, the delay observed between addition of the hormone and onset of cellular response is independent of the insulin concentration when supramaximal hormone concentrations are employed. With less insulin, the time lag is prolonged but this does not correlate with the hormone concentration. This suggests that the delayed response to the insulin signal is unrelated to the speed of hormone–receptor interaction.

At supramaximal concentrations above 100 μU/ml, in spite of a further increase in the association rate, the lag-phase remains constant at ~40 s. Receptor occupancy for a maximal response is reached within the first 10 s if one considers the fact that < 5% of receptors have to be filled [11,12]. Therefore the delay in signal transfer can be viewed as a sign of an additional reaction step between formation of the insulin–receptor complex and initiation of membrane functions. Although there is a difference in dose–response relationship between glucose utilization on the one hand, and antilipolysis on the other [1], the same length in the lag-phase for 3-*O*-methylglucose transport and the lowering of cAMP suggests a common initial mechanism. This reaction appears to be temperature dependent.

In studying equilibrium exchange of 3-*O*-methylglucose with high insulin concentrations (1 μM) an 'absolute lag time' of 50–60 s was described [13] which is close to the value reported herein, but which was much longer at insulin concentrations in the physiological range.

A number of reports suggest that phosphorylation processes [14,15] and oxidation of sulfhydryl groups [16] take place in the plasma membrane as initial steps in the action of insulin. It is hoped, that modification of the lag-phase may yield further information regarding the reactions between insulin binding and activation of membrane functions.

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