ATP MONITORING IN HUMAN RED BLOOD CELLS WITH LUCIFERASE INTRODUCED INTRACELLULARLY

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The simplest, quickest and most sensitive method to measure ATP concentration is that using the light-emitting luciferin-luciferase system of the fireflies.[1,2] As with all other methods it requires cell rupture. Here the suitability of luciferin-luciferase system for ATP monitoring in whole cells is studied.

MATERIALS AND METHODS

Lyophylized hind body segments of fireflies *Luciola mingrelica* were used as a source of luciferase. The segments of 50 fireflies were homogenized in 5 ml of 0.1 M phosphate buffer (pH 7.5), containing 5 mM $MgCl_2$. The homogenate was kept for 30 minutes to extract luciferase. Then it was filtered and mixed with 20 ml of cold lysing solution containing 5 mM glucose and 2 mM ATP. Five ml of washed human erythrocytes were added to this mixture and stirred well. After 1 minute, 30 ml of a solution containing 5 mM glucose, 2 mM ATP, 1 mM $MgCl_2$, 300 mM NaCl, 20 mM sodium phosphate (pH 7.5) were added. The mixture was incubated for 30 minutes at 37° C. Then the loaded erythrocytes were washed and resuspended up to a hematocrit of 30 - 40% in isotonic buffered solution containing glucose (10 mM).

The luciferase loaded erythrocyte suspension was stored at room temperature throughout the experiments (2 - 3 hours). The ATP concentration and luciferase activity in the erythrocytes remained stable during this period. The ATP concentration in the erythrocytes was measured by modified luciferin - luciferase method.[2] Before the luminescence measurements 1-2 ml of luciferase loaded erythrocyte suspension diluted up to desirable hematocrit value were placed into thermostatlly controlling measuring cells and incubated 3 min for temperature equilibration. Then luciferin was added and

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luminescence was measured. All measurements were performed at 37° C with continuous stirring.

Luciferin was obtained from "Serva". It was added to the erythrocyte suspension as a 0.1 mM or 1.0 mM solution in 0.02 M phosphate buffer (pH 7.5) containing 1 mM $MgCl_2$.

RESULTS

Luminescence appeared immediately after luciferin addition to the erythrocyte suspension. Its intensity rose with time, plateaued and then began to decline slowly. Initial rate of the luminescence increase and maximal (at plateau) luminescence intensity of erythrocytes rose along with luciferin concentration in the suspension (Fig.1). At luciferin concentrations lower than 1 μmol/l suspension the maximal luminescence intensity demonstrated a linear relationship on the luciferin concentration. Plateau duration was related to luciferin concentration. As luciferin concentration increased from 1 to 50 μmol/l suspension plateau duration shortened from several minutes to 1-0.5 min. Removal of the cells from luminous suspension (by centrifugation) eliminated luminescence. Luminescence was elevated when hematocrit of the suspension was increased at given luciferin concentration. ATP addition to the suspension (up to 2 mmol/l susp.) did not influence on the luminescence intensity. We concluded that the luminescence observed was caused by intracellular luciferin-luciferase complex and was not related to the luciferase adsorption at the surface of erythrocytes. After the incubation medium was substituted for that without luciferin a luminescence decrease was observed (Fig.2). These data suggest that luciferin can penetrate easily through erythrocyte membrane in both directions. Using data obtained in experiments with the replacement of incubation medium or erythrocytes at low luciferin concentrations it was shown that luciferin distributed uniformly between the incubation medium and the cells.

There are two main factors which determine kinetics of luminescence intensity after luciferin addition to the luciferase loaded erythrocytes. First, is the rate of luciferin transport into erythrocytes, second is the dependence of luciferase reaction on luciferin concentration.

Maximal luminescence intensity under given luciferin concentration seems to correspond to quasi-stationary rate of luciferase reaction when there is an equilibrium between extra- and intracellular luciferin concentrations. The dependence of the maximal luminescence intensity on luciferin concentration is described by Michaelis kinetics (Fig.3). However, the values of Michaelis constants for luciferin differ greatly from experiment to experiment. (Table 1).

The dependence of the initial rate of luminescence increase on the initial luciferin concentration in the medium reflects the kinetics of luciferin transport into erythrocytes. Using data obtained under low luciferin concentrations one can correlate the rate of luminescence increase to the rate of luciferin influx. Determined in this way dependence of initial luciferin influx rate on the initial concentration of luciferin in the medium appeared to be linear (Fig.4). The rate constant values of luciferin transport into erythrocytes measured in different experiments are presented in Table 1.

Temperature dependence of luminescence in luciferase loaded erythrocytes is presented in Table 2. The elvation of maximal luminescence intensity seems to reflect an increase in luciferase activity with temperature rise. To study temperature dependence of luciferin transport rate into erythrocytes one should know the initial velocities of luminescence intensity increase scaled by maximal values of luminescence intensity. As seen in Table 2 luciferin transport rate and maximal luminescence intensity of erythrocytes grow exponentially with temperature increase.

Table 1. Michaelis constant values (K_m) for luciferin and rate constants of luciferin influx (K_1) and efflux (K_2) for the luciferase loaded erythrocytes of different donors

No	K_m (μM)	K_1 (1/sec l cell)	K_2 (1/sec l cell)
1	11.4 ± 0.7	0.014 ± 0.002	0.021
2	8.9 ± 1.8	0.009 ± 0.001	0.014
3	21.5 ± 4.5	0.021 ± 0.003	0.026
4	4.1 ± 0.4	0.011 ± 0.001	0.016-0.022
5	5.4 ± 1.1	0.012 ± 0.002	0.017
6	10.9 ± 1.2	0.014 ± 0.002	0.019

Table 2. Temperature dependencies of maximal luminescence intensity (I_{max}), initial rate of luminescence increase (V) and luciferin influx (V/I_{max}) obtained with luciferase loaded erythrocytes

Temperature (C°)	I_{max} (rel.units)	V (rel. units/sec)	V/I_{max} (1/sec)
23	23.5	0.1	0.0042
30	48	0.35	0.0072
37	72	1.04	0.0144

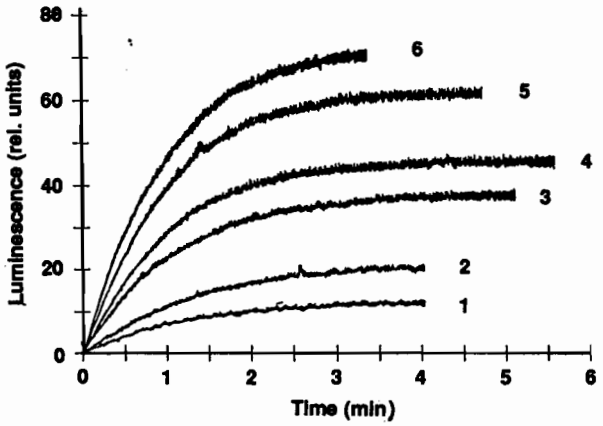

Figure 1. Kinetics of luciferase loaded erythrocytes luminescence after luciferin addition. Hematocrit 5%, luciferin (μmol/l susp.): 1 -0.83, 2 -1.64, 3 -3.23, 4 - 4.76, 5 -6.25, 6 -7.69.

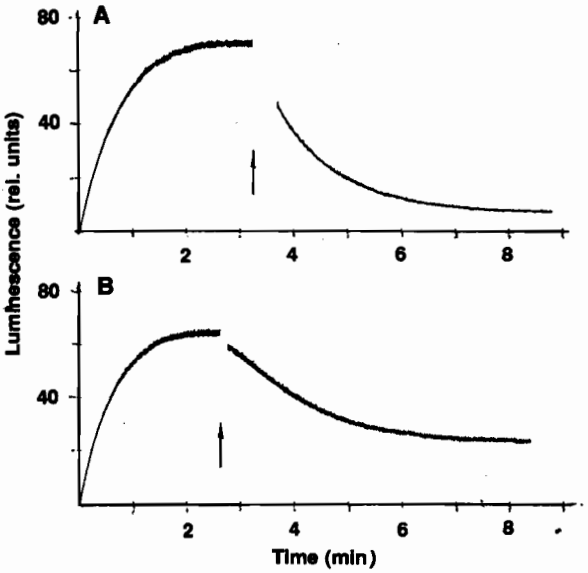

Figure 2. Luciferin outflow from erythrocytes after incubation medium replacement. A - hematocrit 5.8%, luciferin 5.38 μmol/l susp. B - hematocrit 32%, luciferin 0.83 μmol/l susp. Arrows indicate the moment of medium replacement.

For ATP monitoring a freshly prepared suspension of luciferase loaded erythrocytes was divided into four portions and incubated at 37° C with continuous stirring. The first portion was supplemented with glucose (10 mmol/l susp.), the second with glucose (10 mmol/l unit susp.) and luciferin (10 μmol/l unit susp.), the third - with luciferin (10 μmol/l unit susp.) only. The forth portion of the suspension with added luciferin (10 μmol/l unit susp.) was kept for 45 min to deplete intracellular ATP and then glucose (10 mmol/l unit susp.) was added. During the period of incubation several aliquotes were taken from each portion and luminescence intensity was measured, as well as intracellular ATP concentration. Maximal intensities of luminescence in aliquotes from the first portion were measured after preliminary addition of luciferin at final concentration of 10 μmol/l unit susp.

The results are presented in Fig.5. The pattern of changes in ATP concentration of luciferase loaded erythrocytes during ATP depletion in the absence of glucose and during the ATP recovery after glucose addition is very similar to that of the intact normal erythrocytes.[3,4] Interestingly, the ATP depletion proceeds faster in luciferase loaded erythrocytes than in the intact cells. Luminescence intensity of the erythrocytes is continuously decreasing during the long incubations. Nevertheless, the ratio of the luminescence intensities for different portions of the suspension is in qualitative agreement with the corresponding ATP concentration ratio for the portions under examination. This is shown best if relative values are compared when [ATP] and luminescence intensity values for control erythrocytes (incubated with glucose and luciferin) are taken as 100%. In all experiments the luminescence intensity of erythrocytes incubated without luciferin is higher than that of the cells kept with luciferin during incubation. Presence of luciferin in the incubation medium did not affect intracellular ATP concentration.

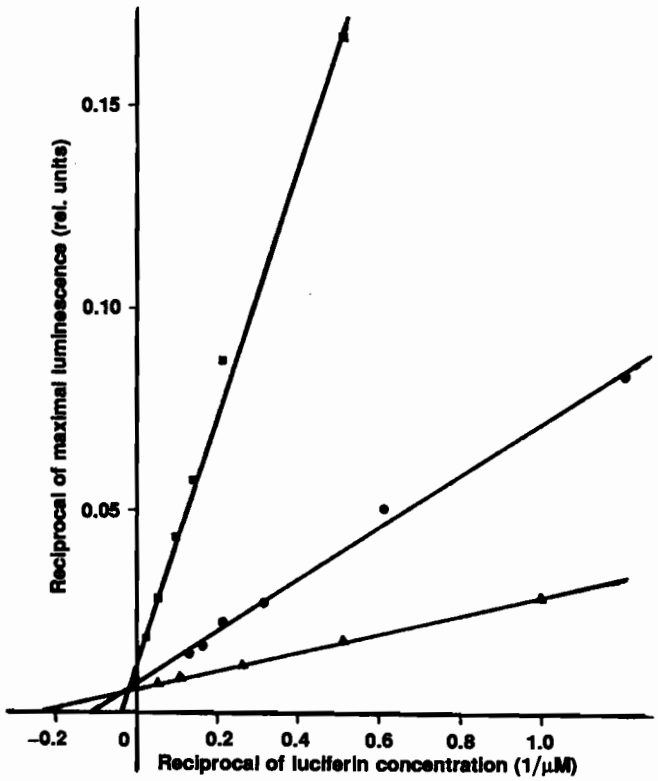

Figure 3. The reciprocal plot of the loaded erythrocyte maximal luminescence intensity versus luciferin concentration. Data of three experiments are shown.

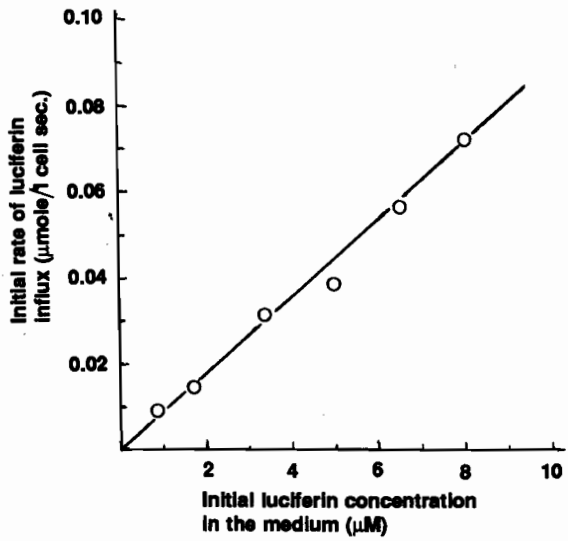

Figure 4. An example of the dependence of the initial rate of the luciferin flux into erythrocytes on the luciferin concentration in the medium.

DISCUSSION

The results obtained show that luciferin can freely penetrate the erythrocyte membrane. Luciferin transport across the erythrocyte membrane seems to be a result of a simple diffusion. Several facts support this conclusion: a linear dependence of initial transport rate on luciferin concentration, free penetration of luciferin through erythrocyte membrane, uniform intra- and extracellular luciferin distribution, exponential dependence of luciferin transport rate on temperature. Furthermore, the assumption that luciferin passively diffuses through the erythrocyte membrane leads to the prediction that the following equation should be satisfied (at low values of hematocrit and low luciferin concentrations provided that extracellular luciferin concentration can be taken as constant and luminescence intensity depends linearly on intracellular luciferin concentration):

$$\ln(1 - I/I_{max}) = - K_2 * t \qquad (1)$$

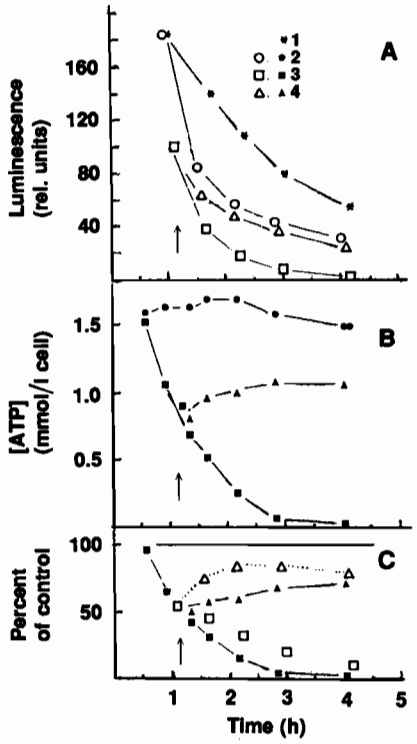

Figure 5. Relationship between intracellular ATP concentration (black symbols) and luciferase loaded erythrocyte luminescence (open symbols) during the incubation at 37°C at hematocrit 8%.
1 - erythrocyte incubation with glucose in the absence of luciferin. Luciferin was added immediately before the measurements and the maximal luminescence was registered;
2 - incubation in the presence of both glucose and luiferin (control); 3 - glucose depleted erythrocytes; 4 - glucose depleted erythrocytes after glucose addition. A moment of the addition is pointed out by an arrow. Open symbols correspond to luminescence intensity, black ones - ATP concentrations. Final concentration of luciferin - 10 μmol/l susp., glucose - 10 mmol/l susp. Fig. 5C presents a comparison of relative luminescence intensities and ATP concentrations normalized to their control value.

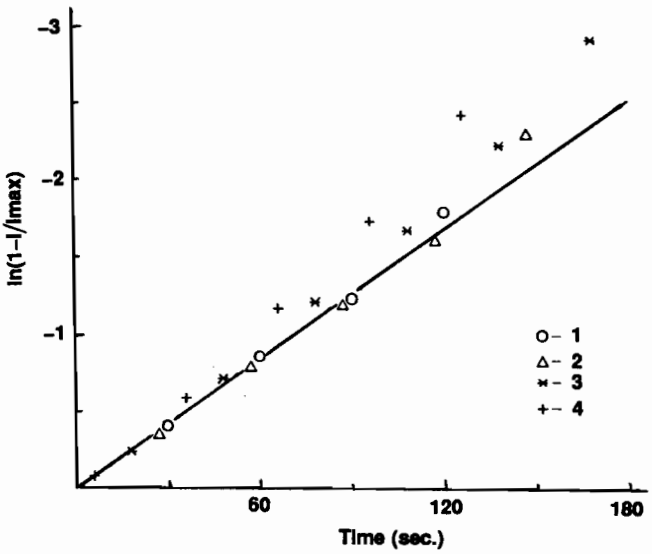

Figure 6. The time dependence of the $\ln(1-I/I_{max})$. Different symbols are enumerated as the curves in Fig. 1 from which the presented data are calculated.

where t is time elapsed from the moment of luciferin addition, I - luminescence intensity of erythrocytes at moment t, I_{max} - maximal luminescence intensity observed when extra- and intracellular luciferin concentrations reach an equilibrium, K_2 - rate constant of luciferin outflow.

The results obtained are in a good agreement with the equation (1). Fig. 6 depicts the dependencies of $-\ln(1 - I/I_{max})$ on t calculated from data presented in Fig. 1. These dependencies begin to deviate from linearity with an increase in luciferin concentration (Fig. 6). Obviously the deviation is related to the non-linear increase of luminescence intensity with luciferin increase. K_2 value can be determined as a slope of the approximation straight line presented in Fig. 6. These values for each experiments are shown in Table 1. K_1 and K_2 values obtained in the same experiment are rather close, K_1 being always less than K_2. Since luciferin is uniformly distributed between cells and an external medium, K_1 and K_2 are expected to be equal. The differences observed might be explained by the fact that different methods have been used for K_1 and K_2 determination.

The study presented demonstrates a principal possibility to monitor [ATP] changes in erythrocytes with a luciferin - luciferase system introduced intracellularly. It is shown that long incubations result in a significant decrease in the luminescence of luciferase loaded erythrocytes although ATP level does not change (Fig.5). This decrease in luminescence could occur for two reasons. Firstly, an inhibition by some product of luciferase reaction could cause it. This inhibition would explain sharp difference in luminescence of erythrocytes incubated with and without luciferin observed at the beginning of the incubation (Fig. 5 A). Secondly, luciferase entrapped could gradually loose its activity during the incubation at 37° C as a result of heat inactivation. It would explain the observed decrease in luminescence intensity throughout the experiments when the luciferase loaded erythrocytes were incubated without luciferin or at the latest stages of incubation with luciferin.

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